# SMALL MOLECULE INHIBITORS OF STEROID RECEPTORS FOR BREAST AND PROSTATE CANCER

BY

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DISSERTATION

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## ABSTRACT

Steroid hormone receptors play a critical role in the growth and progression of hormone-dependent cancers. This thesis aimed at identifying and characterizing new chemical entities with therapeutic potential in breast and prostate cancer treatment. Reported here are two inhibitors that antagonize androgen receptor (AR) function and one that suppresses estrogen receptor (ER) signaling. Moderate-to-high throughput screens of chemical libraries were designed and implemented to identify these inhibitors.

The most promising inhibitor of AR transactivation to have emerged from the rigorous screening process was CPIC (1-(3-(2-chlorophenoxy) propyl)-1H-indole-3-carbonitrile). CPIC is capable of potently and specifically reducing androgen-induced proliferation and gene expression in prostate cancer cells. CPIC also inhibited recruitment of androgen-bound AR to the DNA regulatory sites of target genes in multiple cell lines. CPIC exhibits a mode of action different from that of classical AR antagonists, bicalutamide or flutamide. Additionally, CPIC at nanomolar concentrations, but not bicalutamide, disrupts the amino-carboxyl (N/C) terminal interaction of AR, which is crucial for optimum androgen-mediated AR signaling.

Also described here is a novel non-competitive inhibitor of AR action, AR54 (2-(pyrimidin-2-ylthio)-1-(2,2,4-trimethyl-4-phenyl-3,4-dihydroquinolin-1(2H)-yl)ethanone). AR54 blocks prostate cancer cell proliferation by reducing AR mRNA levels and down-regulating AR protein levels. We have not yet determined whether AR54 reduces the production of AR transcripts or increases the rate of mRNA degradation. AR54 modestly inhibits both androgen-dependent and androgen-independent proliferation of 22Rv1 cells, a model for castration-recurrent prostate cancer.

These novel small molecule inhibitors represent important new probes for understanding AR action. In addition, CPIC and its structural analogues have properties that make them suitable for further evaluation and development as therapeutics for aggressive late-stage prostate cancers resistant to current therapies.

*To my Father, Cherian K Isaac,*

*and Mother, Rosy Cherian.*

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"*Let us be grateful to the people who make us happy; they are the charming gardeners who make our souls blossom.*" ― Marcel Proust

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--Milu

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AF | Activation function |
| AR | Androgen receptor |
| AR54 | 2-(pyrimidin-2-ylthio)-1-(2,2,4-trimethyl-4-phenyl-3,4-dihydroquinolin-1(2H)-yl)ethanone |
| ARE | Androgen response elements |
| Bic | Bicalutamide (Casodex) |
| CD-FBS | Charcoal-dextran stripped fetal bovine serum |
| ChIP | Chromatin immunoprecipitation |
| CPIC | 1-(3-(2-chlorophenoxy)propyl)-1H-indole-3-carbonitrile |
| CRPC | Castration resistant prostate cancer |
| DBD | DNS binding domain |
| Dex | Dexamethasone |
| DHT | Dihydrotestosterone |
| DMSO | Dimethyl sulfoxide |
| $E_2$ | 17-β-estradiol |
| $EC_{50}/IC_{50}$ | half-maximal effective/inhibitory concentration |
| EGF | Epidermal growth factor |
| ER | Estrogen receptor |
| ERE | Estrogen response elements |
| FAMA | Fluorescence anisotropy microplate assay |
| FBS | Fetal bovine serum |
| GR | Glucocorticoid receptor |
| HeLa13 | HeLa-AR3A-PSA-ARE-Luc-13 |
| HeLaA6 | HeLa-AR1C-PSA-ARE-Luc-A6 |
| HTS | High-throughput screening |
| ITS | Insulin-Transferrin-Selenium |
| LBD | Ligand binding domain |
| MMTV | Mouse mammary tumor virus |
| NR | Nuclear receptor |

| | |
|---|---|
| NTD | amino-terminal domain |
| OHF | Hydroxyflutamide |
| OHT | 4-hydroxytamoxifen |
| PIC19.7 | Methyl-1-(3-(o-tolyloxy)propyl)-1H-indole-3-carbonitrile |
| PCa | Prostate cancer |
| PR | Progesterone receptor |
| PSA | Prostate specific antigen |
| qRT-PCR | quantitative reverse transcription polymerase chain reaction |
| R1881 | Methyltrienolone, a synthetic androgen |
| R1881-AR | AR bound to R1881 |
| RPol II | RNA polymerase II |
| SARM | Selective androgen receptor modulator |
| SERM | Selective estrogen receptor modulator |
| SEM | Standard error of the mean |
| TPBM | Theophylline, 8-[(benzylthio)methyl]-(7CI,8CI) |
| TPSF | p-fluoro-4-(1,2,3,6,-tetrahydro-1,3-dimethyl-2-oxo-6-thionpurin-8-ylthio) |
| Z'-factor | A measure of statistical effect size ranging from 0-1 |

# CHAPTER 1
# BACKGROUND

**A**ndrogen and estrogen receptors are key members of the steroid receptor family and unusual in their ability to stimulate cell growth in response to their respective steroid hormones. Since the discovery of the first steroid hormone receptor by Jenson and colleagues (1), this class of nuclear receptors has been understood to be key to life on earth as we know it. The steroid family of receptors comprise mainly of the androgen, estrogen, progesterone, glucocorticoid and mineralocorticoid receptors. Of these, androgen and estrogen receptors have been identified to be unusual in their ability to stimulate cell growth in response to binding their respective steroid hormones. This has great implications in the understanding of normal and diseased physiology.

**Androgen Receptor** (AR) is a Class I member of the nuclear receptor (NR) superfamily of ligand-inducible transcription factors. This receptor contains an N-terminal transactivation domain (NTD, encoded by exon 1), the DNA binding domain (DBD, exons 2 and 3), a short hinge region (exon 4), and the C-terminal ligand-binding domain (LBD, exons 4–8) where the androgenic ligands testosterone and dihydrotestosterone (DHT) bind (Illustrated in Figure 1.1). When bound by androgens, AR undergoes a conformational change that permits nuclear translocation, DNA binding, and regulation of AR target genes (2). Whereas estrogen receptor interacts with coregulators via contact points in the LBD, AR interaction with coregulators occurs primarily via regions of the NTD (3). Along with influencing transcriptional regulation, the N-terminal domain also binds to the C-terminal domain in a phenomenon unique to the AR known as its N/C interaction (4). This intra-molecular interaction plays a role in the stabilization of the AR dimer complex and in stabilization of the ligand-AR complex by slowing the rate of ligand dissociation and decreasing receptor degradation. Androgens like testosterone and DHT induce the N/C interaction in full length AR, unlike antagonists like hydroxyflutamide (OHF) or bicalutamide (Bic). The N/C interaction is altered when AR is specifically bound to response elements on DNA, allowing coregulator binding (5). AR is a transcription factor that up or down regulates target genes in response to androgens (genomic action), facilitated largely by direct interactions between the AR DBD and androgen-responsive elements (ARE) at DNA regulatory

sites of androgen responsive genes. AR also has significant extranuclear functions resulting in transcription-independent steroid signaling.

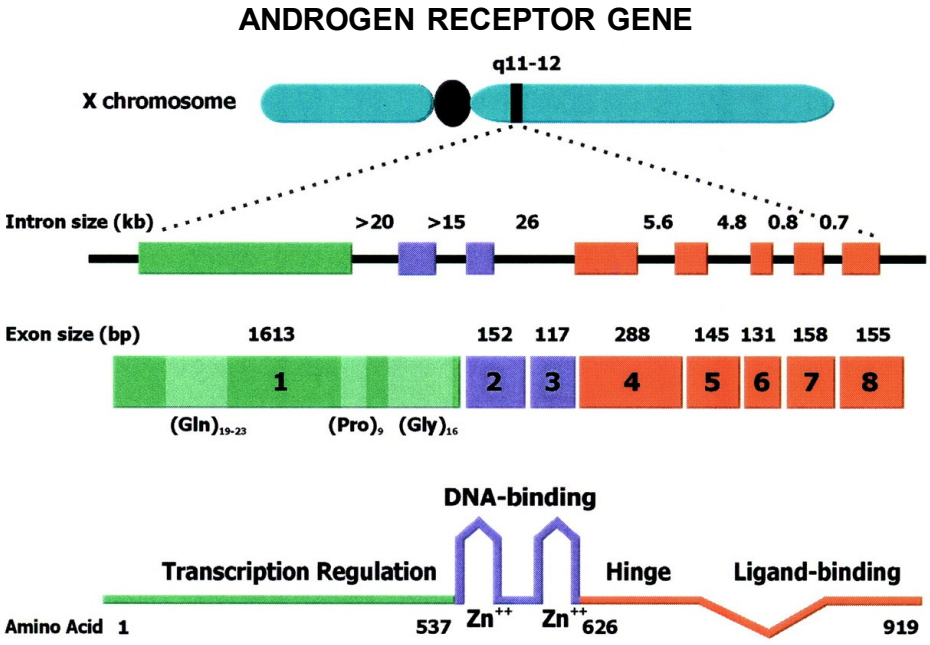


FIGURE 1.1. **Schematic representation of the domain and exon structure of androgen receptor gene**. (*Adapted from Gelmann E P, Journal of Clinical Oncology, 2002; 20.)*

AR signaling is required for development of the normal prostate. In this tissue, the AR mediates androgenic signals that primarily arise from the testes and adrenal glands. Testosterone passively diffuses across the prostate cell membrane, where it is then converted to the more active metabolite DHT by the 5α-reductase enzyme. DHT binds to the AR LBD with high affinity, inducing conformational changes that lead to activation of the receptor. Extracellular peptide signals, such as growth factors and cytokines, can promote AR activity although not through the same ligand-binding mechanism as androgens.

AR signaling is also required for development and progression of prostate cancer, even in the advanced stage of castration-resistant prostate cancer (CRPC) (6). CRPC may develop due to many causes including, (a) changes in the level of ligand in tumor tissue or presence of extra-testicular androgens (7); (b) increased levels of the AR protein due to gene amplification or expression of splice variants (7-11); (c) activating mutations in the receptor that affect structure and function (12-14); (d) changes in coregulatory molecules including coactivators and

corepressors; and (e) factors that lead to activation of the receptor through cross-talk with kinases. Impeding AR signaling remains the main therapeutic objective. New drugs currently in clinical trials (15, 16) may offer modestly improved management of CRPC through antagonism of AR activity or blockage of extra-testicular androgen production. Ultimately, the identification of drugs that promote selective AR degradation may have the greatest impact on the continued action of AR in CRPC (8).

**Estrogen Receptor alpha** (ERα) is also a steroid hormone receptor belonging to Class I of the NR superfamily of ligand-inducible transcription factors (9). The interaction of ERα with target gene promoters either can occur directly, through specific estrogen response elements (EREs), or indirectly through contacts with other DNA bound transcription factors such as AP1, SP1, or NF-κB. Once tethered to DNA, the receptor can either activate or repress target gene transcription by communicating with the general transcription apparatus (Fig 1.2). Two acidic activation domains mediate the ligand-dependent transcriptional activity of ERα, a constitutive activation function-1 (AF-1) in the N-terminus, and a hormone-dependent AF-2 located in the LBD with binding sites for co-regulator proteins. The ER N-terminus is able to activate gene transcription without ligand, but this activation is weak and more selective compared to the activation provided by the LBD. In addition to the above-described classic or 'genomic' ER action, a portion of the ER in the cell may initiate more rapid cellular signaling by direct interaction with components of growth factor signaling pathways in what is referred to as the 'non-genomic' or extranuclear action of ER. The extranuclear actions of ER associated with cell-surface membrane can be activated by estrogens as well as by selective estrogen-receptor modulators (SERMs) such as tamoxifen and raloxifene. Estrogenic compounds are classified as full agonists (estradiol), mixed antagonists (tamoxifen) or pure antagonists (ICI 182780), and this function is decided by ER interactions with various co-regulatory proteins in different tissue types.

ERα action is essential for normal growth and development and is expressed in most physiological systems at low to moderate levels, with highest expression levels observed in the ovary, uterus, breast and pituitary gland. ERα is a key regulator of the cellular processes involved in the development and progression of a majority of breast cancers.

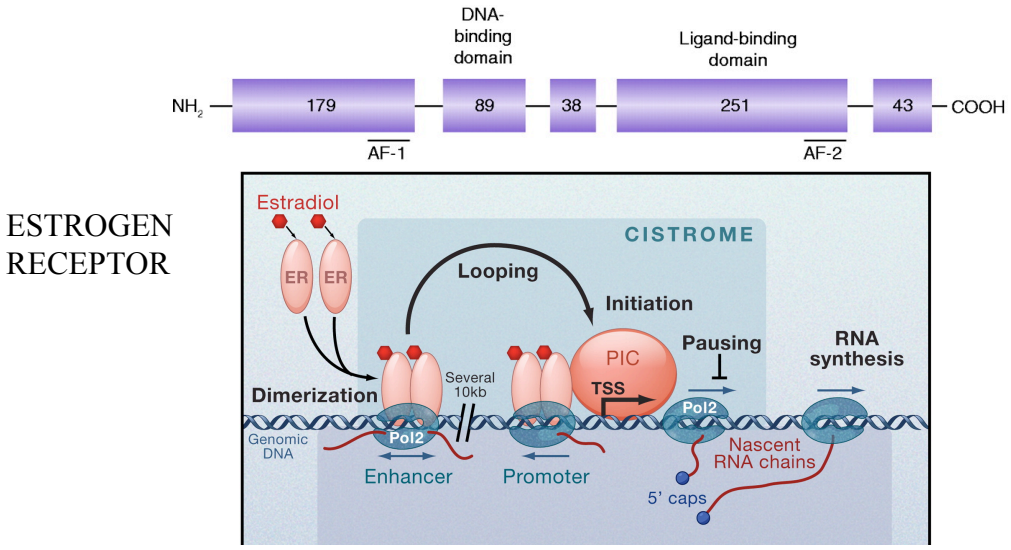


FIGURE 1.2. **ER action at a responsive gene.** *(Adapted from A Prokesch and MA Lazar, Cell, 2011, 145:4, 499-501)*

**Inhibitors of ERα and AR action in Breast and Prostate Cancer -** The central roles played by estrogen receptor α (ERα) and by androgen receptor (AR) in receptor dependent cases of breast and prostate cancer led to an intense effort to identify agents that modulate receptor activity. The availability of substantial information on the interaction of agonist ligands with ERα and AR led to a primary focus on identification of small molecules that act by competing with natural hormones for binding in the ligand-binding pocket of the receptors. This approach was followed by development of agents that act by inhibiting key enzymes in estrogen or androgen synthesis (aromatase inhibitors). While these approaches to development of clinically useful agents remain productive, as shown by recent development of an improved competitive ligand for androgens (MDV3100) (15) and an inhibitor of androgen production in prostate tumors (Abiraterone) (16), their current potential for illuminating novel mechanisms of ERα and AR action is limited.

Our lab intended to focus on small molecule modulators of ERα and AR activity that act outside of the ligand-binding pocket. Some of the sites targeted in attempts to antagonize ER action in breast cancer are illustrated in Figure 1.3, and can be extrapolated to AR as well. In addition to direct interactions with various ligands, both receptors have an array of macromolecular interaction partners that can influence receptor activity (10) including DNA

binding sites, proteins that tether receptors to DNA, coactivators and corepressors, chaperones, ubiquitin ligases, and diverse modifiers including kinases, phosphatases, methylases and acetylases. These interactions provide a wealth of targets that are being exploited by screens to identify small molecules that modulate ERα and AR activity (20-24).

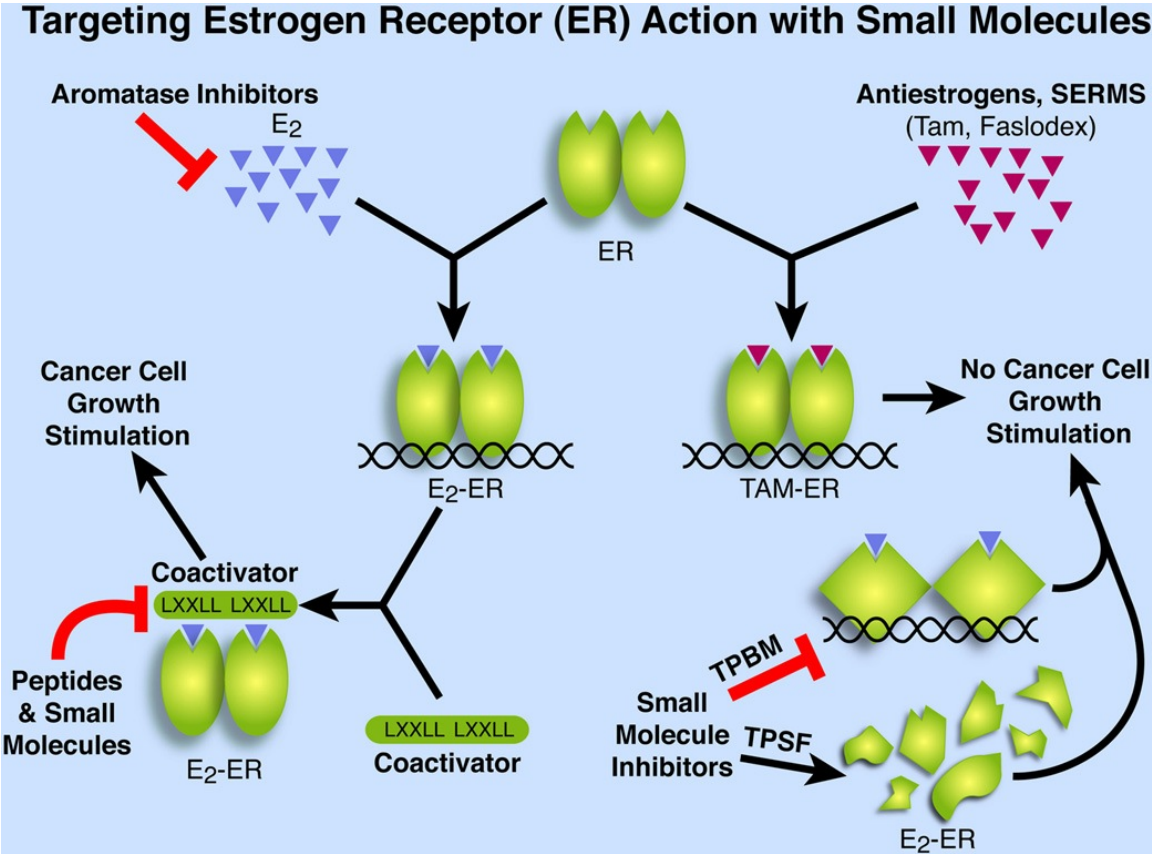


*Figure 1.3:* ***Schematic representation of some sites targeted by small molecules used to selectively block ERα action and breast cancer cell growth.*** *DJ Shapiro et al., J Biol Chem, 2011, 286:4043-8*

In considering efforts to identify small molecule modulators of ERα and AR, it is important to understand why it has been simpler to target binding of natural hormone ligands than to target other sites critical for receptor activity. The natural ligands are relatively small, and bind with very high affinity (low nM to sub nM) in a discrete binding pocket whose 3-dimensional structure has been solved. In contrast, most other ERα and AR interactions involve relatively large, often low affinity, macromolecular interfaces for which little or no structural information is available. Most current non-competitive small molecule modulators of ERα and AR were identified using screens based on known activities of the receptors, such as DNA binding or

coactivator binding. Although binding sites for many of these small molecules are, as yet, unknown, they target well-defined biological processes.

Our lab has been involved in efforts to identify new small molecule inhibitors of AR and ERα action. We employed high-throughput screens (HTS) for this purpose and used different approaches for the two receptors. For ERα, we used a fluorescence anisotropy based microplate assay (FAMA) (25-27) to identify small molecule inhibitors that disrupt ERα binding to ERE containing DNA probes. This HTS identified TPBM as an inhibitor of ER action (20) and through lead optimization, we hit upon TPSF as a non-competitive inhibitor of ERα working at least in part by increasing ERα degradation (11). TPSF is described in Chapter 2 of this document.

Although we performed a FAMA-based small-scale screen for identifying AR inhibitors, we found that there was poor translation of effective small molecules from *in vitro* assays to cell-based assays for the AR. This motivated us to develop a luciferase-based screen for AR HTS. For this screen we used HeLa cells stably transfected to express high amounts of AR and a luciferase reporter under control of the prostate specific antigen (PSA) promoter sequence. We reasoned that using a cell line with high levels of AR protein and natural hormone, dihydrotestosterone (DHT), would help exclude weak-to-moderate competitive antagonists that inhibit the receptor by binding at its ligand-binding pocket. The screening methodologies I used are elaborated in Chapter 3.

We are currently evaluating the properties of some leads from the AR HTS. Two lead inhibitors are described in detail in later chapters; **CPIC (AR19)**, an antagonist of N-terminal AR activity, and **AR54**, a non-competitive small molecule that reduces AR transcripts.

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# CHAPTER 2

# A NONCOMPETITIVE SMALL MOLECULE INHIBITOR OF BREAST CANCER CELL GROWTH THAT ENHANCES PROTEASOME-DEPENDENT DEGRADATION OF ESTROGEN RECEPTOR ALPHA[1¶]

The mechanisms responsible for 17β-estradiol ($E_2$)-stimulated breast cancer growth and development of resistance to tamoxifen and other estrogen receptor α (ERα) antagonists are not fully understood. We describe a new tool for dissecting ERα action in breast cancer, p-fluoro-4-(1,2,3,6,-tetrahydro-1, 3-dimethyl-2-oxo-6-thionpurin-8-ylthio) (TPSF), a potent small-molecule inhibitor of estrogen receptor α, that does not compete with estrogen for binding to ERα. TPSF noncompetitively inhibits estrogen-dependent ERα-mediated gene expression with little inhibition of transcriptional activity by NF-κB, or the androgen or glucocorticoid receptor. TPSF inhibits $E_2$-ERα-mediated induction of the proteinase inhibitor 9 (PI-9) gene, which is activated by ERα binding to estrogen response element DNA and the cyclin D1 gene which is induced by tethering ERα to other DNA-bound proteins. TPSF inhibits anchorage-dependent and anchorage-independent $E_2$-ERα stimulated growth of MCF-7 cells, but does not inhibit growth of ER-negative MDA-MB-231 breast cancer cells. TPSF also inhibits ERα-dependent growth in 3 cellular models for tamoxifen resistance: 4-hydroxytamoxifen-stimulated MCF7ERαHA cells that overexpress ERα, in fully tamoxifen-resistant BT474 cells that have amplified HER-2 and AIB1, and in partially tamoxifen-resistant ZR-75 cells. TPSF reduces ERα protein levels in MCF-7 cells and several other cell lines, without altering ERα mRNA levels. The proteasome inhibitor MG132 abolished down-regulation of ERα by TPSF. Thus, TPSF effects receptor levels at least in part due to its ability to enhance proteasome-dependent degradation of ERα. TPSF represents a novel class of ER inhibitor with significant clinical potential.

[1] This research was originally published in Journal of Biological Chemistry. 2010, 285(53): 41863-73. I thank my co-authors for their significant contributions to this work.

[¶] Disclosure: The University of Illinois at Urbana-Champaign has filed a novel use patent that claims TPSF.

## INTRODUCTION

Estrogen receptor α (ERα) is a well-studied member of the steroid/nuclear receptor family of transcription regulators. ERα acts in the nucleus to regulate gene expression by binding to estrogen response elements (EREs) and related DNA sequences (1-4) and through association with transcription factors bound at SP1 and AP-1 DNA binding sites (4-7). In response to high affinity estrogen binding, ERα dimerizes, binds to ERE DNAs and undergoes a conformational change in the ligand-binding domain that facilitates the recruitment of coactivators (8). Bound coactivators promote assembly of a multiprotein complex that enables chromatin remodeling and stabilization of an active transcription complex (9-11). In contrast, antagonist-occupied ERα recruits corepressors (12).

At detection, growth of most human breast cancers depends on 17β-estradiol ($E_2$) binding to ERα (13-16). Treatment strategies that inhibit estrogen-dependent breast cancer include selective ER modulators such as tamoxifen that binds in the ERα ligand-binding pocket, and aromatase inhibitors that block estrogen production. Nearly half of patients treated with aromatase inhibitors develop resistance (17). The long-term effectiveness of tamoxifen is limited by the development of resistance in nearly all patients with metastatic breast cancer and in ~40% of patients with primary breast cancers (18). The development of resistance to current therapies underscores the need to develop new small molecule antagonists that act outside the ligand-binding pocket of ERα. We recently described an *in vitro* high throughput screening strategy to identify small molecule inhibitors of ERα binding to DNA. We identified 8-benzylsulfanylmethyl-1,3-dimethyl-3,7-dihydro-purine-2,6-dione (TPBM) as a small molecule inhibitor of ERα binding to ERE DNA (19). Using a cell-based screen, we evaluated approximately 200 small molecules structurally related to TPBM, and identified butyrophenone, p-fluoro-4- (1,2,3,6, -tetrahydro-1, 3-dimethyl-2-oxo-6-thionpurin-8-ylthio) (TPSF) as a novel inhibitor of ERα >15-fold more potent than TPBM. Although structurally related to TPBM, TPSF exhibits an entirely different mode of action. While TPBM inhibits *in vitro* binding of $E_2$-ERα to a labeled ERE, TPSF does not. TPSF strongly reduces ERα levels in breast cancer cells, while TPBM has little or no effect on the level of ERα. Here we demonstrate the selectivity of TPSF and its ability to inhibit expression of an endogenous ERα regulated gene that contains EREs and a gene regulated by tethering of ERα through other proteins. We show that TPSF

inhibits anchorage-dependent and anchorage-independent growth of tamoxifen-sensitive and tamoxifen-resistant ERα-containing breast cancer cells and demonstrate that TPSF enhances proteosome-dependent degradation of ERα.

## EXPERIMENTAL PROCEDURES

***Cell Culture***- Unless otherwise indicated, cells were maintained at 37˚C in 5% $CO_2$ in growth medium containing 1% penicillin and streptomycin and fetal bovine serum (FBS) (Atlanta Biological, Atlanta, GA) or calf serum, and transferred to phenol-red free medium containing charcoal-dextran (CD) stripped serum at least 2 days prior to treatment with $E_2$, 4-hydroxytamoxifen (OHT) or TPSF. ERα positive MCF-7 and ER-negative MDA-MB-231, human breast cancer cells, were cultured in MEM supplemented with 10% calf serum and switched to MEM containing 5% CD-treated calf serum 3 or 4 days before the experiment. The medium was changed on day 2. Tet-inducible MCF7ERαHA cells were maintained in DMEM supplemented with 1 mM sodium pyruvate, 0.5 μg/ml puromycin and 10% FBS. Four days before the experiment, MCF7ERαHA cells were switched to the above medium without phenol red containing 10% 6X stripped CD-treated FBS, without puromycin (20-23). ZR-75 human breast cancer cells were maintained in MEM containing 10% calf serum and transferred to medium containing 10% CD-CS 4 days before the experiment. BT474 human breast cancer cells were maintained in improved MEM (iMEM) containing 10% FBS and transferred to phenol red-free iMEM containing 10% CD-FBS 4 days before the experiment. T47D-KBluc breast cancer cells expressing an $(ERE)_3$-luciferase reporter gene (24) were maintained in phenol red-free RPMI 1640 containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM Hepes, pH 7.5, 1 mM sodium pyruvate, 10% FBS. Four days before induction with $E_2$, cells were transferred to medium without phenol red, containing 10% 2X CD calf serum. T47D/A1-2 cells that stably express the glucocorticoid receptor (GR) and contain a mouse mammary tumor virus (MMTV)-luciferase reporter (25) were maintained in MEM supplemented with 10 mM HEPES, pH 7.4, 2 mM glutamine, 5% FBS and 0.2 mg/ml geneticin (G418). Four days before the experiment the cells were transferred to the above phenol red-free medium (phenol red- free) containing 10% 2X CD CS. HeLa-AR1C-PSA-Luc-A6 cells that stably express AR and a prostate-specific antigen (PSA)-Luc reporter were maintained in phenol-red free MEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% FBS under selection with

0.1 mg/ml hygromycin B (Roche) and 0.5 mg/ml G418. Four days before the experiment cells were transferred to medium containing 10% 2X CD-CS.

***Fluorescence Anisotropy Assays***- The fluorescence anisotropy microplate assay for analyzing binding of ERα to the fluorescein-labeled consensus ERE was as described (19).

***Competitive Radioligand Binding Assays***-The relative binding affinity of TPSF for ERα and ERβ was determined in competitive radioligand binding assays using 2 nM $^3$H-$E_2$ and a range of TPSF concentrations as described (26,27).

***Reporter Gene Assays***-Reporter gene assays were performed to compare the ability of about 200 compounds structurally related to TPBM (19) to inhibit estrogen-dependent transcription in T47D-KBluc breast cancer cells stably transfected to express an $(ERE)_3$-Luc reporter (24). The ability of TPSF to inhibit AR and GR transcriptional activity was assayed in HeLa-AR1C-PSA-Luc-A6 cells that stably express human AR and a PSA-Luc reporter, and in T47D/A1-2 cells that stably express GR and MMTV-Luc. Four days before each experiment, cells were switched to medium containing CD-treated serum as described above. HeLaAR-PSA-Luc cells (100,000 cells/well) and T47DA/1-2 and T47D-KBluc cells (200,000 cells/well) were plated in 1 ml of media in 24-well plates. After 24 h the indicated concentrations of $E_2$, dihydrotestosterone (DHT) or dexamethasone (Dex) in DMSO, or DMSO vehicle alone, with or without TPSF, were added to each well. After 24 h, cells were washed once with phosphate buffered saline and lysed in 100 μl of Passive Lysis Buffer (Promega, Madison WI). Luciferase activity was determined using BrightGlo firefly luciferase reagent from Promega (Madison, WI).

***Endogenous Gene Expression***- MCF-7 cells and MCF7ERαHA cells were maintained for 4 days in medium containing 5% 1X CD CS (MCF-7 cells) or 10% 6X stripped CD FBS (MCF7ERαHA cells). For assays of TPSF inhibition of PI-9 induction in MCF-7 cells, cells were preincubated for 24 h with TPSF and then maintained for 4 h with and without $E_2$ and TPSF, or with vehicle alone. To induce ERα expression, MCF7ERαHA cells were maintained in medium containing 0.5 μg/ml doxycycline (Dox) for 24 h. $E_2$ or OHT was added with or without TPSF and maintained for 24 h. For the induction of cyclin D1, 24 h after plating the cells, $E_2$ with and without TPSF was added and cells were maintained for 24 h. RNA was extracted and mRNA levels were measured by quantitative RT-PCR as described (19,28). Actin mRNA level is used as the qRT-PCR internal standard. Primers used in qRT-PCR were: ERα, Forward-5'GGAGACGGACCAAAGCCACT, Reverse- 5'TTCCCAACAGAAGACAGAAGATG;

Cyclin D1, Forward- 5'TCATGGCTGAAGTCACCTCTTGGT, Reverse- 5'TCCACTGGATGGTTTGTCACTGGA; PI-9, Forward- 5'TGGAATGAACCGTTTGACGAA, Reverse- 5'CATCTGCACTGGCCTTTGCT; IL-8' Forward-5'GAGGGTTGTGGAGAAGTTTTTG, Reverse- 5'CTGGCATCTTCACTGATTCTT G; β-actin, Forward- 5'AAGCCACCCCACTTCTCTCTAA, Reverse- 5'AATGCTATCACCTCCCCTGTGT.

***Cell Growth and Viability Assays*-**Cells were maintained in CD-treated serum for at least 4 days prior to each experiment. To minimize cell aggregation, MCF-7 cells were harvested in 10 mM HEPES, pH 7.4, 1 mM EDTA. Other cell lines were harvested in trypsin-EDTA. To assay anchorage-dependent cell growth, 1,000 cells/well were plated in a 96 well plate. For slow growing ZR-75 cells, 2,000 cells were plated/well. Cells were maintained in medium containing CD-treated serum for 24 h and the medium was then changed and $E_2$ and DMSO vehicle or TPSF in DMSO was added. The medium was replaced after 2 days, except for BT474 cells whose medium was not changed. After 4 days, cell viability was determined using Promega CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison WI).

To assay anchorage-independent cell growth in soft agar, 1% and 0.7% Select Agar (Invitrogen), were prepared in water and warmed at 40°C before use. 1.5 ml of 0.5% bottom agar diluted in medium was added to each well of a 6-well cell culture plate and allowed to solidify at room temperature. Top agar was prepared by dilution in warm medium containing the various treatments. MCF-7 cells were resuspended in 1.5 ml of 0.35% top agar at 5,000 cells/well and plated in 3 wells for each condition. The plate was kept at room temperature for 30 min until the top agar solidified, then 0.5 ml of medium containing the respective treatments was added on top of the agar. Culture medium on top of the agar was changed every 3-4 days. Colonies were visible by 1 week and counted at day 16 using a dissecting microscope. Photographs of colonies were taken using a Zeiss AxioImager2 imaging system at 6X magnification.

***Western Blot*-**MCF-7 cells were plated at 200,000 cells/well in 6-well plates in MEM containing 5% 1X CD FBS. The medium was changed at day 2, and at day 4 the medium was replaced with fresh medium containing the indicated treatments. Whole cell extracts were prepared after 24 hours in 1XRIPA buffer (Millipore, CA) containing protease inhibitor cocktail (Roche, Germany). Extract (20 µg protein/lane) was run on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. ER was detected using a 1:4,000 dilution of ERα antibody ER6F11

(Bio Care Medical, CA). The blot was stripped for 25 min and reprobed using a 1:10,000 dilution of β-actin monoclonal antibody (Sigma, Saint Louis MO).

***Statistical Analysis***-Results are expressed as mean ± SEM of at least three independent experiments. Student's t test was used for comparison of the means between two groups. Significance was established when $p<0.05$. The comparisons are described in the figure legends and are not shown in the body of the figures.

**RESULTS**

***TPSF is a Structure-specific Inhibitor that Acts Outside of the Ligand-binding Pocket of ERα***-We evaluated the ability of approximately 200 compounds structurally related to TPBM (Fig 2.1A), a first generation ERα inhibitor (19), to inhibit $E_2$-ERα-mediated gene expression in ERα-positive T47D-KBluc human breast cancer cells containing $(ERE)_3$-Luc (24). Of the compounds tested, TPSF was ~16 fold more potent than TPBM in inhibiting ERα mediated gene expression (Fig 2.1B).

To test whether TPSF is a structure-specific inhibitor, we compared the ability of TPSF to inhibit ERα mediated gene expression to the structurally similar small molecule 99676. TPSF differs from NSC 99676 by having hydrophilic C=O and F substitutions at the phenyl ring (Fig 2.1A). The similar, but more hydrophobic, 99676 had an $IC_{50}$ of 9 μM, and is ~13 fold less potent than TPSF (Fig 2.1B). The results suggest that TPSF is a structure-specific inhibitor of ERα and is not simply acting by promiscuous inhibition due to micelle formation.

If TPSF inhibited ERα by competing with $E_2$ for binding in the ligand-binding pocket of the receptor, increasing the $E_2$ concentration should reduce the ability of TPSF to bind ERα and block its action. To test this, we varied the $E_2$ concentration by 500 fold and tested the ability of TPSF to inhibit $(ERE)_3$-Luc in T47D cells. Increasing the concentration of $E_2$ from 0.2 nM to 100 nM only slightly increased the $IC_{50}$ for inhibiting $E_2$-ERα-mediated transcription from 0.4 μM; (Fig 2.1B, filled triangles), to 0.7 μM (Fig 2.1B, open triangles), suggesting TPSF acts outside the ERα ligand-binding pocket.

If TPSF is a highly potent ERα ligand, it might retain the ability to inhibit ERα at 100 nM $E_2$. To test whether TPSF binds in the ligand-binding pocket of ERα, the ability of TPSF to compete with radiolabeled $E_2$ for binding to ERα was evaluated. In competitive radiometric

binding assays performed across a broad range of concentrations (26,27), TPSF had virtually no ability to compete with $E_2$ for binding to ERα. With $E_2$ set at 100%, TPSF had a relative binding affinity for ERα of ~0.001%, indicating that it is not a classical ligand that competes with $E_2$ for binding in the ERα ligand-binding pocket.

***TPSF is a Specific Inhibitor of ERα Transactivation***-The ERα binding cleft for p160 coactivator LxxLL motifs has been a major target for development of peptide and small molecule inhibitors (29-33). Although these inhibitors are effective in reporter gene assays in transfected cells, in general, they have not been shown to effectively inhibit expression of endogenous ER-regulated genes in breast cancer cells. We therefore tested the ability of TPSF to inhibit the expression of the endogenous $E_2$-inducible proteinase inhibitor 9 (PI-9) gene in MCF-7 cells. The serpin PI-9 is a tumor lethality factor (34-36) whose induction by estrogens enables breast cancer cells to evade apoptosis induced by the immune cells, cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (23,28,37). PI-9 also inhibits tumor necrosis factor-α (TNF-α) Fas and TRAIL mediated apoptosis (38,39). Induction of PI-9 results from direct binding of $E_2$-ERα to EREs and ERE half sites (19,40,41). TPSF ($IC_{50}$= 0.2 μM) potently inhibited $E_2$-ERα-stimulated induction of PI-9 mRNA (Fig 2.2A).

To examine the ability of TPSF to inhibit $E_2$-ERα induction of a gene regulated by tethering of $E_2$-ERα through DNA-bound transcriptional regulators, we tested the effect of TPSF on induction of cyclin D1 mRNA. Cyclin D1 is thought to contribute to the growth of MCF-7 and other breast cancer cells (42-44) and to tamoxifen-stimulated growth of breast cancer cells (45). As previously reported (43,46), $E_2$-ERα stimulated a 2-3 fold increase in cyclin D1 mRNA, that was blocked by 10 μM TPSF (Fig 2B). The data demonstrate that TPSF inhibits $E_2$-dependent gene expression through mechanisms that include direct binding of ERα to EREs and through tethering of ERα to DNA-associated transcription regulators.

Specificity of TPSF inhibition of $E_2$-ERα mediated gene expression was evaluated by comparing the ability of TPSF to inhibit gene expression mediated by NF-κB (Fig 2.2C) and by other steroid receptors (Fig 2.2D). The NF-κB-regulated IL-8 gene was used to test specificity because many regulators and pathways including IκB and other kinases, the ubiquitin/proteasome pathway and nuclear/cytoplasmic shuttling all influence NF-κB activity (47). Analyzing the effect of TPSF on NF-κB is a good way to test whether TPSF acts as a

promiscuous inhibitor targeting diverse cell proteins and pathways. 30 μM TPSF had no effect on the NF-κB-mediated induction of IL-8 mRNA by TNF-α (Fig 2.2C). In the same breast cancer cells where TPSF inhibited $E_2$-ERα induction of PI-9 mRNA (Fig 2.2A, $IC_{50}$=0.2 μM), a >100 fold higher concentration of TPSF had no effect on NF-κB-mediated induction of IL-8 mRNA by TNF-α (Fig 2.2C).

To further evaluate the specificity of TPSF, we compared the ability of TPSF to inhibit ERα to its effect on the androgen receptor (AR) and glucocorticoid receptor (GR)-mediated activation of stably transfected reporter genes. AR was assayed in HeLa cells stably transfected to express AR and a prostate specific antigen (PSA)-luciferase reporter. TPSF only very weakly inhibited DHT-AR-mediated induction of the PSA-Luc reporter ($IC_{50}$=33 μM, Fig 2.2D). GR was assayed in T47D cells stably transfected to express GR and an MMTV-Luc reporter (25). TPSF weakly inhibited GR activation of the MMTV-luc reporter in T47D cells ($IC_{50}$=10 μM). Although T47D cells contain substantial levels of the progesterone receptor (PR), cross-talk between ERα and PR makes them unsuitable for assaying inhibitor specificity using PR. The ER antagonist faslodex/fulvestrant/ICI 182,780 inhibited PR induction of the endogenous alkaline phosphatase gene (Supplemental Fig S2.1). TPSF did not inhibit NF-κB and concentrations of TPSF required to inhibit AR (33 μM) and GR (10 μM) are far higher than the 0.2 and 0.4 μM TPSF required to inhibit the endogenous PI-9 gene and the stably transfected $(ERE)_3$-Luc reporter. At low concentrations, TPSF is a relatively specific ERα inhibitor.

***TPSF inhibits $E_2$ and OHT-induced Gene Expression in Tamoxifen-stimulated MCF7ERαHA Cells***-Development of resistance to tamoxifen, and other therapeutics that target ERα and estrogen production results in treatment failure in both primary and metastatic breast cancer. Recent studies show that tamoxifen-resistant breast cancer cells that retain dependence on ERα for growth lose their dependence on SRC3 and other p160 coactivators for $E_2$-ERα mediated gene transcription (22,48). We explored the ability of TPSF to inhibit $E_2$ and OHT-dependent gene expression in tamoxifen-resistant cells that are less dependent on p160 coactivators for transactivation.

MCF7ERαHA cells are an MCF-7-breast cancer cell line engineered to increase ERα expression in response to Doxycycline (Dox) (20,22). In Dox-induced MCF7ERαHA cells overexpressing ERα, tamoxifen and 4-hydroxytamoxifen (OHT) are potent ERα agonists (22,23)

and increase ERα-mediated gene expression independent of SRC3 (22). Because OHT stabilizes ERα against degradation, while $E_2$ down-regulates ERα (23,37), ERα levels are ~4 times higher in OHT treated MCF7ERαHA cells than in cells treated with $E_2$. The elevated level of ERα in OHT-treated MCF7ERαHA cells compared to cells treated with $E_2$ renders OHT more effective than $E_2$ in inducing PI-9 gene expression and more difficult to inhibit. 10 μM TPSF inhibited $E_2$-ERα (Fig 2.3A) and OHT-ERα induction of PI-9 mRNA (Fig 2.3B). This indicates that TPSF is an inhibitor of both $E_2$-ERα and OHT-ERα mediated gene expression in cells where tamoxifen is a full agonist.

***TPSF Inhibits Estrogen-dependent Growth of MCF-7 Cells and Exhibits Low Toxicity in ERα Negative MDA-MB-231 Cells***-To determine whether TPSF specifically inhibits ERα-dependent growth of breast cancer cells with minimal nonspecific cell toxicity, we tested TPSF inhibition of cell growth in MCF-7 cells and ERα negative MDA-MB-231 human breast cancer cells. Compared to MCF-7 cells in estrogen-depleted medium, both 1 pM and 10 pM $E_2$ stimulated a 4-5 fold increase in cell number after 4 days (Fig 2.4 and data not shown). TPSF elicited a dose-dependent inhibition of estrogen-dependent growth of MCF-7 cells ($IC_{50}$= 2 μM) and completely blocked $E_2$-dependent growth at 7.5 μM (Fig 2.4, filled circles). However, TPSF did not inhibit $E_2$-independent cell growth (Fig 2.4, compare 7.5 and 10 μM TPSF, filled circles to no $E_2$ or TPSF, open circle). TPSF did not inhibit growth of ERα negative MDA-MB-231 cells at all concentrations, including 30 μM (Fig 2.4, filled triangles). To rule out the possibility that MDA-MB-231 cells are unusually resistant to TPSF or other ERα inhibitors, we compared the effects of TPSF and OHT on the growth of MDA-MB-231 cells. TPSF was less toxic to ER negative MDA-MB-231 cells than OHT (Supplemental Fig S2.2). The results suggest that TPSF specifically inhibits ERα-mediated growth of breast cancer cells with low nonspecific toxicity in ERα negative cells.

***TPSF Inhibits Anchorage-independent Growth of MCF-7 and BT474 Cells***-The capacity for anchorage-independent growth is a hallmark of cancer cells. Growth in soft agar is often used to evaluate anchorage-independent growth of human breast cancer cells. We tested the ability of TPSF to inhibit $E_2$-stimulated growth of MCF-7 cells in soft agar. MCF-7 cells grown in medium containing $E_2$ formed large colonies after 16 days. Addition of 10 μM TPSF completely inhibited growth of MCF-7 cells in soft agar (Fig 2.5 A). When colonies in equal areas of the soft agar

plate were counted, the $E_2$ treated plate had 30 colonies >0.5 mm in diameter, whereas there were no colonies >0.5 mm in diameter in the $E_2$ and TPSF treated plate. Similar results were obtained with the tamoxifen-resistant BT474 cells as well (Fig 2.5 B). The data indicate that TPSF inhibits estrogen-stimulation of anchorage-dependent (Fig 2.4) and anchorage-independent (Fig 2.5) growth of breast cancer cells.

***TPSF Inhibits $E_2$-ERα-dependent Growth of Tamoxifen-resistant Breast Cancer Cells***-The ability of TPSF to inhibit $E_2$-ERα-dependent cell growth was tested using human cell models of tamoxifen-resistant breast cancer. ZR-75 cells are usually reported as partially tamoxifen and OHT-resistant (49-51), while BT474 cells are fully tamoxifen-resistant and contain amplified expression of HER2 and AIB1 (52,53). TPSF inhibited $E_2$-ERα-dependent growth of BT474 and ZR-75 cells with near maximal inhibition at 5 μM TPSF (Fig 2.6). Since TPSF has minimal nonspecific toxic effects, cell numbers after TPSF treatment were not zero and represented cells plated at day zero plus $E_2$-ERα-independent cell growth over the 4 days. TPSF $IC_{50}$ values were 0.9 μM for slow-growing ZR-75 cells and 1.6 μM for BT474 cells. The lower levels of ERα in ZR-75 compared to MCF-7 cells (54), may be responsible for the greater potency of TPSF in ZR-75 cells. Some tamoxifen-resistant breast cancers regress after tamoxifen withdrawal, suggesting tamoxifen stimulates tumor growth (45,55-57). The MCF7ERαHA cell line is a model for tamoxifen-stimulated breast cancer, where tamoxifen and OHT act as full agonists (Fig 2.3) (23,37). In MCF7ERαHA cells treated with Dox, overexpression of ERα increased $E_2$-independent ERα-mediated cell growth, which was modestly increased by 1 pM $E_2$ with and without 5 μM OHT, and was inhibited by 5 μM TPSF (Supplemental Fig S2.3). Table 1 summarizes the effect of TPSF on gene expression and cell growth.

***TPSF and TPBM have Different Modes of Action***-Our data show that TPSF is a potent and selective inhibitor ER-stimulated gene expression and breast cancer cell growth. We therefore began to assess how TPSF might exert its actions. We used our fluorescence anisotropy microplate assay (FAMA) (19,58,59) to compare the ability of TPSF and TPBM to inhibit binding of purified ERα to a fluorescein-labeled consensus ERE (flcERE). When polarized light excites the flcERE, most of the emitted light is depolarized because of rapid rotational diffusion of the flcERE that results in its position being largely randomized at the time of emission. Binding of the larger ERα protein to the flcERE slows rotation of the flcERE, increasing the

likelihood that the complex is in the same plane at emission and excitation. Interaction of ERα with the flcERE increases fluorescence polarization (FP)/fluorescence anisotropy (FA).

We compared the ability of TPBM and TPSF to inhibit binding of ERα to the flcERE. Consistent with our recent report (19), TPBM inhibited binding of $E_2$-ERα to the ERE (Fig 2.7A). Surprisingly, even at 30 μM, TPSF had no effect on binding of $E_2$-ERα to the flcERE (Fig 2.7A). Thus, in a direct *in vitro* assay containing only $E_2$-ERα, the flcERE, TPSF did not inhibit binding of ERα to an ERE.

We next compared the effects of TPSF and TPBM on the intracellular levels of $E_2$-ERα in MCF-7 cells. TPBM, at 5-20 μM, had little or no effect on the level of $E_2$-ERα. In contrast, TPSF elicited a concentration-dependent reduction in $E_2$-ERα levels, with 10 μM TPSF decreasing the level of $E_2$-ERα by ~4 fold (Fig 2.7B). TPSF also reduced $E_2$-ERα levels in T47D breast cancer cells (Fig 2.7C). Since TPSF had very little or no effect on the levels of AR and GR (Supplemental Fig S2.4), TPSF selectively down-regulates the level of ERα. The results indicate that TPBM and TPSF have distinct modes of action, and that TPSF is not simply a more potent version of TPBM.

***TPSF Does Not Alter the Level of ER mRNA***-TPSF might reduce ERα levels by decreasing transcription, or by destabilizing ERα mRNA. To test for effects of TPSF at the mRNA level, we examined the effect of TPSF on ERα levels in HeLa cells that stably express ERα mRNA from a CMV promoter. TPSF retained the ability to down-regulate ERα protein from the CMV promoter and from the 2 kb ERα mRNA coding region that lacks ~4 kb of 5' and 3' untranslated region (Fig 2.8A). TPSF had no effect ERα mRNA levels in MCF-7 cells (Fig 2.8B). Taken together the results suggest that TPSF down-regulates ERα protein levels through mechanisms that are independent of the level of ERα mRNA.

***The Proteasome Inhibitor MG132 Blocks the Down-regulation of ERα by TPSF***- To further examine the effects of TPSF we determined the time course of TPSF down-regulation of ERα. Consistent with a TPSF-induced increase in proteasome-dependent degradation of ERα, there was a progressive decrease in ERα protein levels after 6-8 h (Fig 2.9A). To examine this further we tested the ability of the proteasome inhibitor MG132 to block the effects of TPSF. Compared to $E_2$ alone, TPSF reduced ER levels, and the TPSF-mediated reduction in ERα levels was completely blocked by MG132 (Fig 2.9B). Efforts to examine the effect of TPSF on ubiquitination of ERα in MCF-7 cells were complicated by the use of endogenous untagged

endogenous ubiquitin and because $E_2$ down-regulates ER and influences its degradation. The data suggests that much of TPSFs effectiveness as an ER inhibitor resides in its ability to enhance proteasome-mediated degradation of ERα.

***TPSF Does Not Enhance ERα Degradation by Reducing the Level of the Muc1 Oncoprotein***- Kufe and coworkers reported that the cytoplasmic domain of the Muc1 oncoprotein binds ERα and stabilizes ERα against degradation; which contributes to enhanced ERα transactivation and the estrogen-dependent growth of breast cancer cells. Although the mechanism by which Muc1 influences ER degradation is unknown, knockdown of Muc1 with RNAi enhanced degradation of ERα, and inhibited ERα-mediated transactivation and growth of ER positive breast cancer cells (60). Because the effects of TPSF and RNAi knockdown of Muc1 protein are similar, we tested whether TPSF influenced the level of the Muc1 cytoplasmic domain. Using the same antibody used by by Kufe and coworkers, TPSF did not alter the level of the Muc1 cytoplasmic domain (Supplemental Fig S2.5), indicating that the reduction in ERα levels elicited by TPSF was likely independent of the level of Muc1.

## DISCUSSION

***Specificity and Toxicity of TPSF***- An optimal small molecule inhibitor of $E_2$-ERα action and growth of breast cancer cells will exhibit specificity for ERα and low nonspecific toxicity. Independent testing of TPSF at concentrations up to 10 μM against a panel of 60 cancer cell lines at the National Cancer Institute Developmental Therapeutics Program demonstrated that TPSF is generally not toxic to cancer cells (testing was terminated because <6 of the 60 cell lines showed 50% inhibition of cell growth at 10 μM TPSF). In agreement with this, we provide evidence that TPSF selectivity targets $E_2$-ERα-dependent cell growth, with little effect on ERα-independent cell growth. After 4-days of treatment, $E_2$ increased MCF-7 cell number by ~4 fold, which corresponds to a doubling time of ~1 day with $E_2$ and ~2 days without $E_2$. The number of cells treated with 7.5 μM and 10 μM TPSF was similar to that seen without $E_2$. In addition, studies using ER-negative MDA-MB-231 cells showed 30 μM TPSF was not toxic. The ability of 10-20 μM OHT to induce apoptosis of MDA-MB-231 cells suggests that these cells are not especially resistant to nonspecific toxic effects. Over several decades, tamoxifen has displayed an excellent safety profile in humans. The toxicity of OHT is used only to demonstrate that MDA-MB-231

cells remain susceptible to cell death and that the failure of TPSF to damage the cells is therefore due to low toxicity, rather than resistance of these cells to cell death.

Several lines of evidence support the specificity of TPSF for ERα. For example, NF-κB is regulated by a variety of signaling mechanisms that include the ubiquitin/proteasome pathway, nuclear/cytoplasmic shuttling, IκB and other kinases and acetylases, (47). In MCF-7 cells, TNF-α activation of NF-κB in MCF-7 cells increases IL-8 mRNA levels by ~50 fold. The absence of an effect of 30 μM TPSF on TNF-α induction of IL-8 mRNA suggests that TPSF does not exhibit nonspecific effects on these diverse cell pathways.

TPSF specificity for ERα was also demonstrated relative to other steroid receptors. TPSF strongly down-regulated the level of ERα, but had very little or no effect on the levels of AR and GR. TPSF was a more potent inhibitor of transactivation by ERα than by AR or GR. We have identified other compounds that inhibit AR and GR under the same assay conditions (data not shown), suggesting that the failure of low concentrations of TPSF to inhibit transactivation by AR and GR was not due to assay conditions. The partial inhibition of GR and AR by higher concentrations of TPSF will not impede future animal or human studies. Two recently described AR inhibitors being tested for prostate cancer therapy, harmol and pyrvinium (61) were strong inhibitors of GR but were used with some success as AR inhibitors in studies in mice (61). Mifepristone (RU-486), a classical PR antagonist that also inhibits GR, has been used in long-term clinical studies without significant GR-related pathology (62,63).

Our initial ER inhibitor, TPBM, has proven useful as a selective inhibitor of the binding of $E_2$-ERα to cellular genes (64,65). The identification of a new coactivator binding surface on AR using moderate potency ($IC_{50}$ ~50 μM) small molecule inhibitors of AR selected by screening (66), that are unrelated to TPSF, also supports the utility of small molecule inhibitors as probes to understand the mechanisms of steroid receptor action.

***Inhibition of Gene Expression by TPSF***- ERα activates gene expression by direct binding to ERE-related DNA sequences and by tethering to DNA-associated transcription factors. Our studies indicate that both of these mechanisms are inhibited by TPSF. TPSF inhibited the induction of PI-9 mRNA by $E_2$-ERα and by OHT-ERα. PI-9 gene expression induced by $E_2$: ERα results from binding to two adjacent ER binding sites in the PI-9 promoter region (40,41). Induction of PI-9 may be a mechanism by which estrogens enable breast cancers to evade

immune surveillance and apoptosis (23,37). PI-9 inhibits granzyme B and cytotoxic T lymphocyte and natural killer and cell mediated apoptosis of target cancer cells (23,28,37), and caspase 8-dependent apoptosis induced by TNF-α-family members (38,39). Expression of PI-9 is associated with a poor prognosis in some cancers (34-36,67).

Cyclin D1 plays a key role in cell cycle progression and is induced by tethering $E_2$-ERα to transcription factors bound at SP1 sites (46). Cyclin D1 induction is proposed to play a role in estrogen-dependent growth of breast cancer cells (42,43,45). Consistent with TPSF as a broad spectrum ERα inhibitor, 10 μM TPSF abolished $E_2$ induction of cyclin D1 mRNA, but did not reduce the level of cyclin D1 mRNA much below the basal (-$E_2$) level. Inhibition of $E_2$-ERα-dependent MCF-7 cell growth by 10 μM TPSF is consistent with a role for cyclin D1 in estrogen-stimulated growth of breast cancer cells. Our work extends earlier studies demonstrating that nearly complete loss of cyclin D1 following RNAi knockdown reduces growth of MCF-7 cells in medium containing estrogen (68). Because TPSF specifically targets the ERα-regulated component of target gene expression, and did not influence basal gene expression, TPSF may be a promising new probe to help clarify the role of ERα regulation of specific genes in growth of breast cancer cells.

***TPSF is Effective in Tamoxifen-stimulated and Tamoxifen-resistant Breast Cancer Cells***- Development of resistance to tamoxifen and other endocrine therapies is a multifactorial clinical challenge in the treatment of breast cancer. A therapeutically useful small molecule inhibitor of ERα should inhibit the growth of a primary tumor as do tamoxifen and its active metabolite OHT, and also inhibit growth of tumor cells with acquired resistance to tamoxifen. Tamoxifen-resistant tumors can be grouped into three broad classes. Some tumors become independent of ERα for growth and may be unaffected by therapies that target ERα. Others tumors remain dependent on $E_2$ and ERα for growth. A third group loses estrogen-dependence but requires ERα for growth. The mechanisms involved in resistance to endocrine therapy are diverse. For example, ERα transactivation in tamoxifen-resistant cell lines may depend on as yet unidentified coregulators, or may be independent of the p160 coactivators (22,48).

One proposed mechanism for tumor resistance to antagonists is the overexpression of steroid receptors. Overexpression of AR was suggested as an important mechanism of resistance to endocrine therapy in castration-recurrent prostate cancer (69). A subset of breast cancers that

contain high levels of ERα are often refractory to tamoxifen therapy (70-72). In MCF7ERαHA cells that overexpress ERα, tamoxifen and OHT are full agonists and induce PI-9 expression. In MCF7ERαHA cells maintained in the presence of OHT, levels of ERα are >10 times higher than in wild-type MCF-7 cells maintained in the presence of $E_2$ (37). In cells expressing high levels of ERα, 10 μM TPSF inhibited both $E_2$-ERα and OHT-ERα induction of PI-9 mRNA.

Clinical specimens of tamoxifen-resistant metastatic breast cancer can be difficult to obtain (73). We and others (49) have therefore evaluated ERα inhibitors using stable breast cancer cell lines resistant to tamoxifen. TPSF inhibited $E_2$-ERα-dependent growth of ZR-75 human breast cancer cells ($IC_{50}$=0.9 μM), whose slow growth is only weakly stimulated by $E_2$ and are partially resistant to tamoxifen and OHT (49,74). In contrast to OHT, TPSF blocked the growth of MCF7ERαHA cells that are tamoxifen-resistant because they overexpress ERα. TPSF inhibited $E_2$-ERα-dependent growth of BT474 cells, which contain amplified HER2 and AIB1 and are fully tamoxifen resistant in cell culture (49) and in xenograft studies (52). Thus, TPSF is effective in cells that become tamoxifen-resistant through different mechanisms.

***Small Molecules Inhibitors of ERα-***TPSF is structurally distinct from disulfide benzamide (DIBA), a zinc-chelator that acts outside the ERα ligand-binding pocket. DIBA promotes an ERα conformation conducive to the antagonist activity of OHT in tamoxifen-resistant cell lines. However, 5 μM DIBA inhibited the growth of ZR-75 cells by ~20% but did not inhibit the growth of tamoxifen-resistant BT474 cells (49). In contrast, growth of both ZR-75 cells and BT474 cells was inhibited by TPSF ($IC_{50}$s=0.9 and 1.6 μM, respectively). Since TPSF does not compete with $E_2$ for binding to ER in a direct binding assay or in transactivation, TPSF is not a classical antagonist ligand and is distinct from known ERα inhibitors.

Fulvestrant is a high affinity ER ligand with nearly pure antagonist activity. Although fulvestrant is used therapeutically to treat advanced breast cancer, its use is limited by the fact that it can require several months for fulvestrant to reach a therapeutic level in serum (75). It has been known for many years that fulvestrant and related compounds, such as ICI 162,380 enhance the degradation of ERα (76,77), although the mechanisms are not known. Recent solution of the structure of fulvestrant bound to the ligand binding domain of ERα suggests that fulvestrant binding may distort ERα structure so that a few hydrophobic amino acids are exposed near the

surface, perhaps triggering recognition of ERα as a misfolded protein and rapid degradation (8). While this is an attractive hypothesis, this idea has not been tested in experiments.

***Structurally-related TPBM and TPSF Elicit Different Effects***- Because ERα and other steroid receptors exhibit a high level of conformational flexibility small molecules can elicit quite different conformations when they interact with ERα. For example, binding of $E_2$ or OHT in the ERα ligand-binding pocket resulted in functionally distinct agonist and antagonist conformations (8). Thus, binding of structurally related ERα inhibitors TPBM (19), and the more potent TPSF, may cause distinct ERα conformations that are associated with different modes of action. The different actions of TPBM and TPSF are illustrated in Fig 2.10. TPBM, inhibited $E_2$-ERα binding to ERE DNA *in vitro*, but had no effect on the intracellular level of ERα. In contrast, TPSF had no effect on binding of $E_2$-ERα to ERE DNA, but elicited a concentration-dependent reduction in ERα protein levels. TPSF also reduced the ERα protein level in our HeLa-ERα cells that stably express FLAG tagged ERα from a cytomegalovirus promoter that drives transcription of the ~2,000 nucleotide ERα cDNA (76) and did not reduce the level of ERα mRNA. Thus, at least part of the inhibitory effect of TPSF appears to reflect its ability to down-regulate ERα protein. $E_2$-ERα induction of PI-9 mRNA and of the stably transfected $(ERE)_3$-Luc reporter is inhibited by TPSF with $IC_{50}$s of 0.2 μM and 0.7 μM, respectively, with only a modest effect on ERα levels. It is possible that regulation of some genes is more sensitive to small changes ERα levels. Another possibility is that low concentrations of TPSF did not saturate ERα. Under these conditions, ERα may assume a transient TPSF-induced conformation sufficient to alter ERα function and inhibit $E_2$-ERα-mediated transactivation at PI-9 and $(ERE)_3$-Luc, whereas higher concentrations of TPSF may be required for an effect on levels of ERα.

In conclusion, TPSF is a potent and specific small molecule inhibitor of ERα that blocks ERα-mediated gene expression and estrogen dependent growth of tamoxifen-sensitive and tamoxifen-resistant human breast cancer cells. TPSF inhibition of ERα is consistent with a role for estrogen induction of cyclinD1 in triggering the growth of breast cancer cells. TPSF represents a novel compound with potential for treating breast cancer and for probing the mechanisms of ER action.

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**TABLES & FIGURES**

**Table 2.1**

Summary of $IC_{50}$ values (μM) for inhibition of gene expression and growth of ERα positive and ERα negative human breast cancer cells*

| | | | | $IC_{50}$(μM) |
|---|---|---|---|---|
| Gene Expression | Reporter assays in T47D stable lines | | ER | 0.7 |
| | | | AR | 34 |
| | | | GR | 10 |
| | qRT-PCR in MCF-7 cells | | Endogenous PI-9 | 0.2 |
| | | | NF-κB | >>30 |
| Cell Growth | ERα positive breast cancer cell lines | Tam sensitive | MCF-7 | 2 |
| | | Partially Tam resistant | ZR-75 | 0.9 |
| | | Tam resistant | BT474 | 1.6 |
| | ER negative cell lines | | MDA-MB-231 | >30 (0% inhibition) |

* $IC_{50}$ values were calculated from data in Figs. 1,2,4 and 6 by curve fitting using SigmaPlot.

**Figure 2.1**

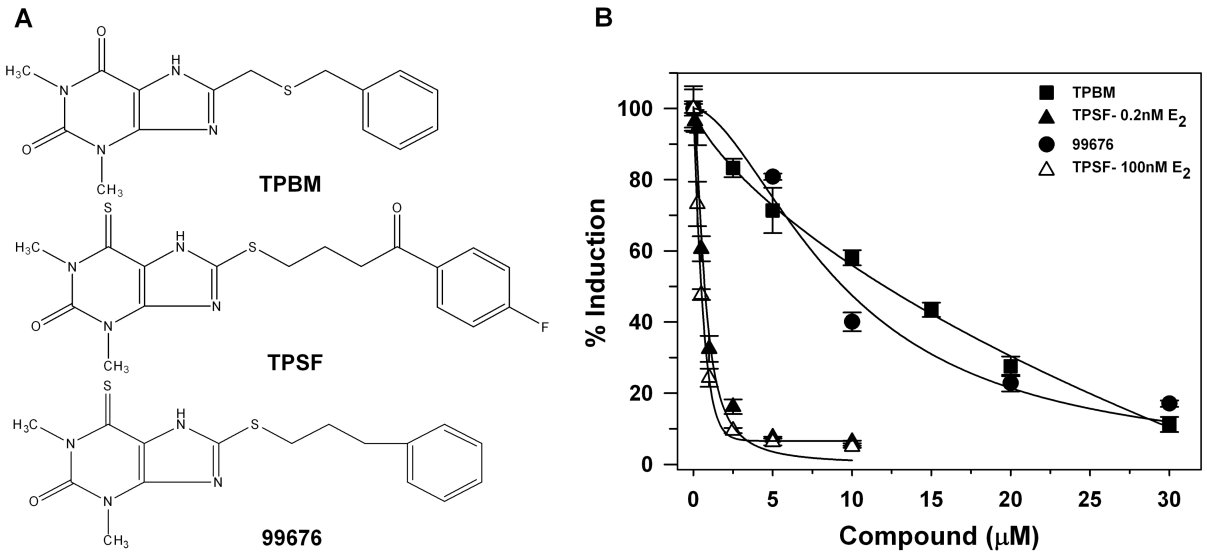



FIGURE 2.1. **Structure-specific inhibition of $E_2$-ERα-mediated gene expression by TPSF**. *A*. Structures of three ERα inhibitors. TPBM is a recently described ERα inhibitor (19). TPSF is butyrophenone, p-fluoro-4-(1,2,3,6,-tetrahydro-1, 3-dimethyl-2-oxo-6-thionpurin-8-ylthio and is known also as theophylline, 8-(3-p-fluorobenzoylpropyl)thio-6-thio-). NSC-99676 is similar to TPSF except TPSF has C=O and F substitutions at the phenyl ring. *B*. Potency and efficacy of TPSF (triangles), TPBM (squares) and 99676 (circles). Inhibition of $E_2$-ERα activation of ERE-Luc was evaluated in dose-response studies of T47D $(ERE)_3$-Luc cells maintained in 0.2 nM $E_2$ (filled triangles, squares and circles) or 100 nM $E_2$ (open triangles) with the indicated concentrations of TPBM, TPSF, or 99676 present for 24 h prior to assay. Activity of the reporter in the presence of the tested concentration of $E_2$ with DMSO and no inhibitor was set to 100%. Data are the average of 3 experiments ± SEM. $IC_{50}$s were calculated by curve fitting using Sigma Plot.

**Figure 2.2**

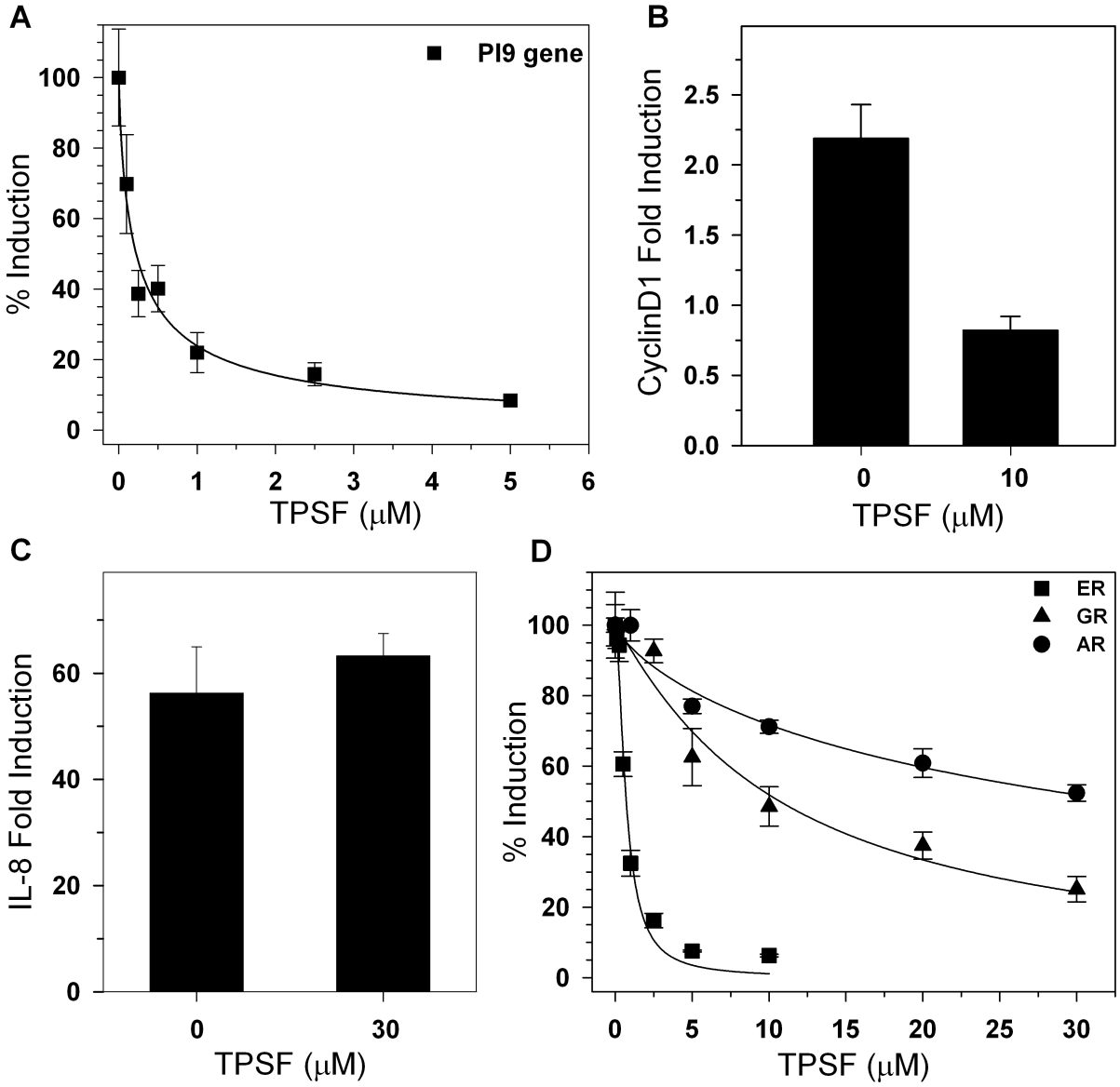



FIGURE 2.2. **TPSF specifically inhibits expression of endogenous ER-regulated genes.** *A*. TPSF inhibits $E_2$ induction of PI-9 mRNA. For studies of PI-9 mRNA (filled squares), MCF-7 cells were incubated for 24 h with the indicated concentrations of TPSF and maintained for 4 h in 10 nM $E_2$ and TPSF. PI-9 mRNA was quantitated by RT-PCR as described (23). *B.* TPSF inhibition of $E_2$-ERα induction of cyclin D1 mRNA. MCF-7 cells were plated and 24 h later treated with ethanol and DMSO vehicles, 10 nM $E_2$, or 10 nM $E_2$ and 10 μM TPSF. After 24 h, RNA was extracted and cyclin D1 mRNA levels measured by qRT-PCR. The level of cyclin D1

mRNA in the vehicle only sample was set to 1. Fold induction of cyclin D1 in the presence of 10 μM TPSF was significantly different from the control ($p < 0.05$ using Student's t test). *C*. TPSF does not inhibit NF-κB induction of IL-8 mRNA. MCF-7 cells were maintained for 24 h in medium without TNF-α or with 10 ng/ml TNF-α with and without 30 μM TPSF, harvested and IL-8 mRNA levels determined by qRT-PCR. *D*. Dose-response studies of inhibition of ERα, AR and GR transactivation. For each receptor, induction of luciferase reporter gene expression (AR and GR), or endogenous PI-9 mRNA (ER), in the presence of an appropriate ligand with DMSO minus TPSF was set to 100%. Cells were incubated for 24 h with 0.2 nM $E_2$ for ERα, (filled squares), 5 nM Dex for GR, (filled triangles), 1 μM DHT for AR (filled circles) and the indicated concentrations of TPSF. Data are the average ± SEM for 3 experiments.

Experimental Data in Figure 2.2 A, B: NM Kretzer

**Figure 2.3**

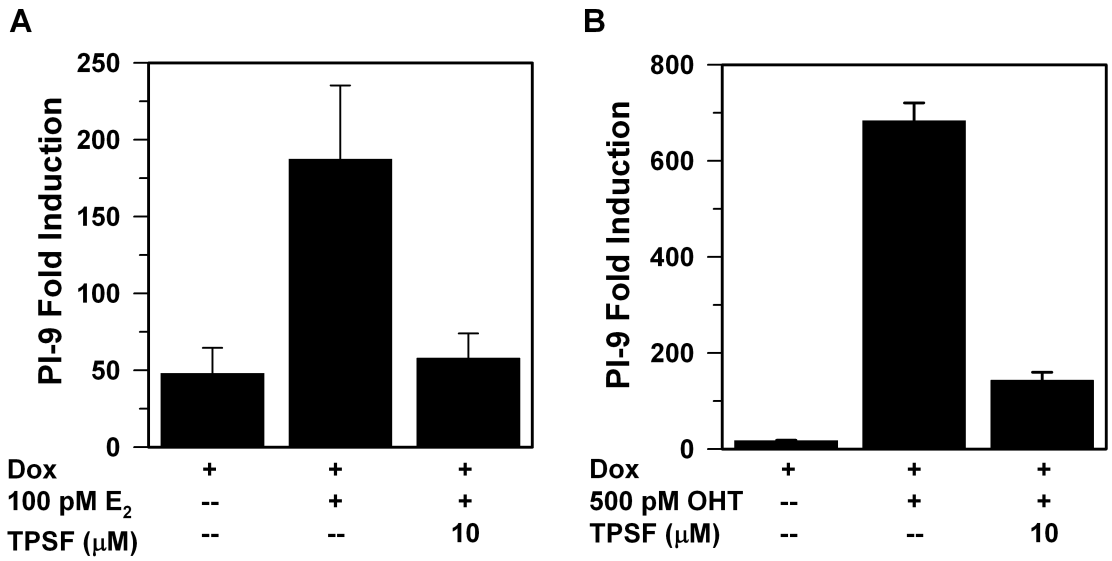



FIGURE 2.3. **TPSF inhibits $E_2$ and OHT-induced gene expression in a tamoxifen-stimulated cell line.** *A* and *B*. MCF7ERαHA cells maintained in 6X CD-FBS (22,37) were treated for 24 h with 0.5 μg/ml Dox to induce ERα expression (37) and *A.* 100 pM $E_2$ or *B*. 500 pM OHT and 10 μM TPSF as indicated. PI-9 mRNA levels were measured by qRT-PCR. PI-9 mRNA in control MCF7ERαHA cells not treated with Dox, $E_2$ or OHT was set equal to 1. The high level of ERα in Dox-treated cells results in ligand-independent transactivation of PI-9 (37). Data are the average, with the range shown, for 2 experiments for $E_2$ and 3 experiments ± SEM for OHT. Experimental Data in Figure 2.3: NM Kretzer

**Figure 2.4**

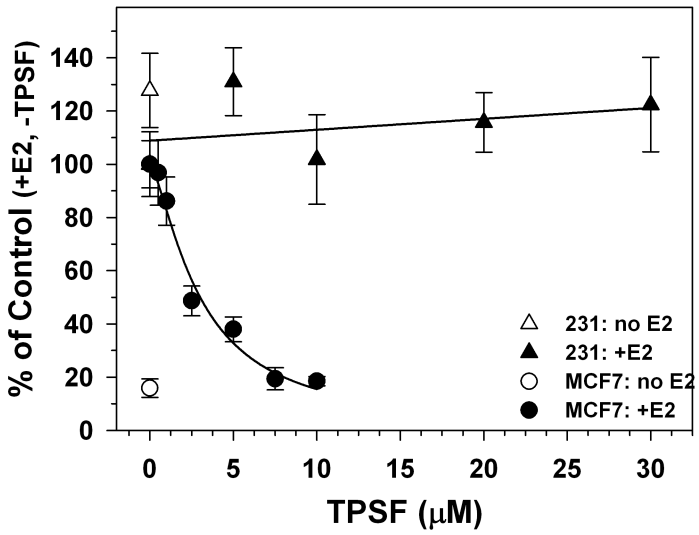



FIGURE 2.4. **Inhibition of $E_2$-ERα-dependent breast cancer cell growth by TPSF.** MCF-7 and MDA-MB-231 cells were maintained for 4 days in 5% CD-CS and 1,000 MCF-7 cells (circles) or MDA-MB-231 cells (triangles) were plated per well in 96 well plates. After 24 h, the medium was changed to 5% CD-CS with 1 pM $E_2$ (filled circles or triangles) or without $E_2$ (open circle and open triangle) and DMSO vehicle and the indicated concentrations of TPSF. Medium was replaced after 2 days and cells were assayed with MTS after a total of 4 days. Cell number was determined using a standard curve of cell number versus absorbance based on plating a known number of cells and assaying using MTS. Each data point is the average of 8 wells ± SEM. The percentage of cells present after 4 days with $E_2$ and without TPSF was set equal to 100. By curve fitting in Sigma Plot, the $IC_{50}$ for inhibition of $E_2$-dependent growth of MCF-7 cells by TPSF was 2 μM.

**Figure 2.5**

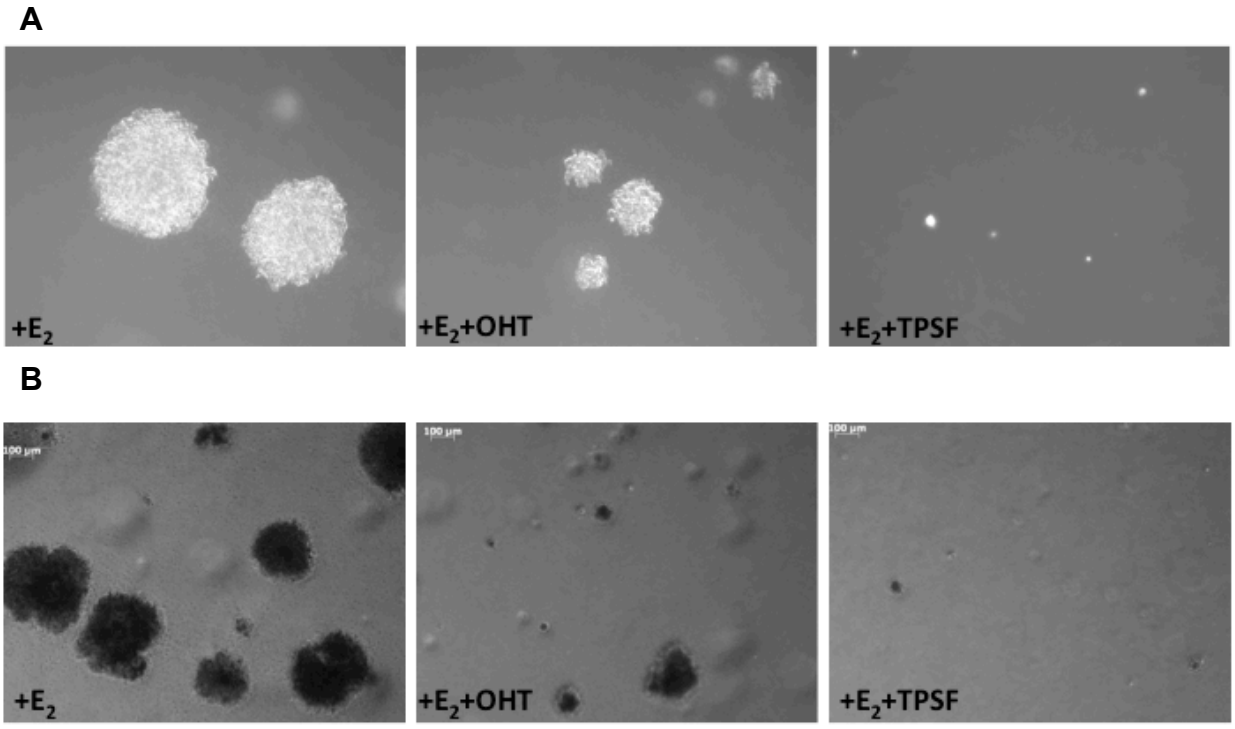



FIGURE 2.5. **TPSF inhibits growth of MCF-7 and BT474 cells in soft agar.** 5,000 MCF-7 cells (A) or BT474 cells (B) were plated into top agar containing 1 pM $E_2$, $E_2$ + 10 μM hydroxytamoxifen (OHT) or $E_2$ + 10 μM TPSF as described in Materials and Methods. After 16 days of culture for MCF-7 cells and 28 days for BT474 cells, colonies were photographed at 5x magnification and counted. Photographs are representative of the entire plate and of duplicate experiments.

**Figure 2.6**

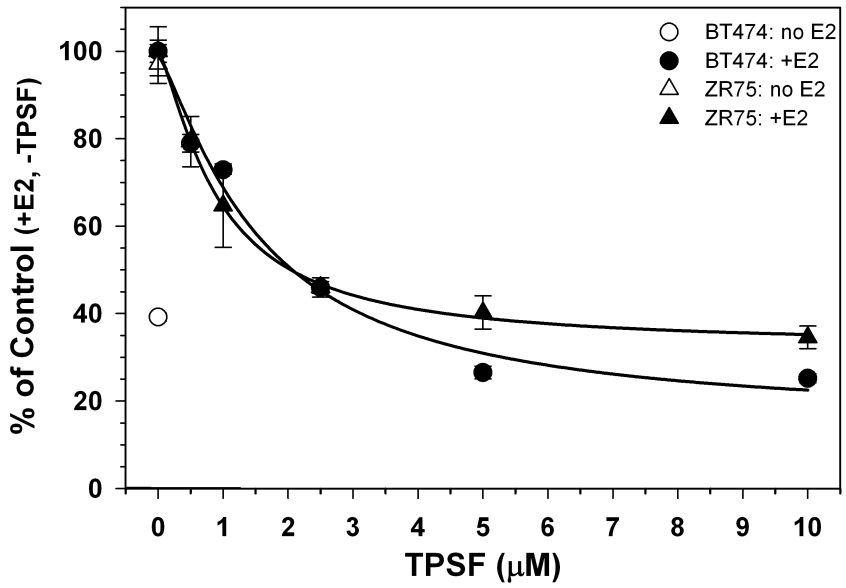



FIGURE 2.6. **TPSF inhibits $E_2$-ERα-dependent growth of tamoxifen-resistant BT474 and ZR-75 cells.** Cells were maintained in medium containing 10% CD-FBS (ZR-75) (triangles) or 10% CD-CS (BT474) (circles) with or without 100 pM $E_2$ and the indicated concentrations of TPSF. Viable cells were measured by comparison to a standard curve of cell number versus absorbance using the MTS assay. Data represents the average of at least 4 wells. $IC_{50}$s for TPSF inhibition of cell growth were calculated by curve-fitting using Sigma Plot. Although some portion of ZR-75 cell growth is likely ERα-independent, to calculate the $IC_{50}$ using Sigma Plot, we used the conservative assumption that all cell growth beyond the 2,000 ZR-75 cells plated was $E_2$-ERα-dependent growth and thus subject to inhibition by TPSF.

**Figure 2.7**

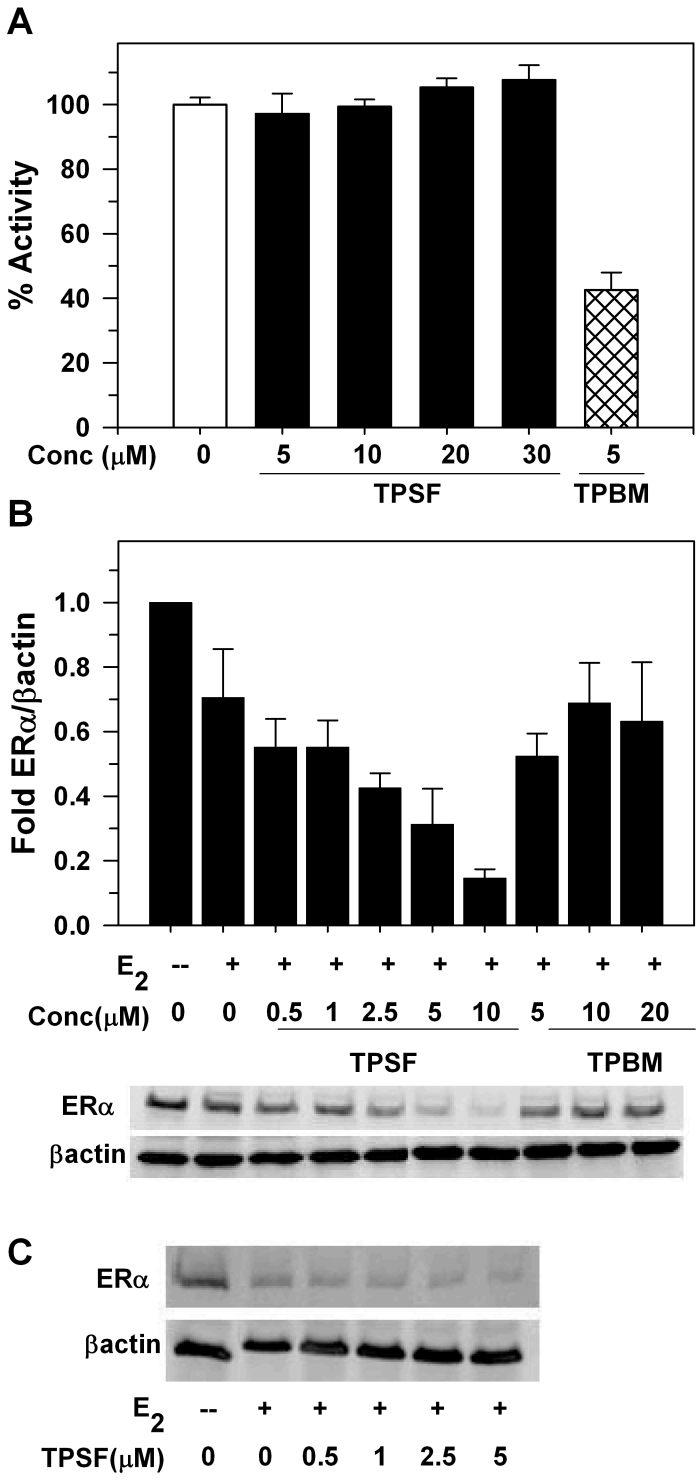



FIGURE 2.7. **Different modes of action of TPSF and TPBM.** *A*. TPSF does not inhibit binding of $E_2$-ERα to the flcERE. FAMA was performed as described (19) in the presence of increasing concentrations of TPSF and 5 μM TPBM. Consistent with our detailed dose-response study (19), 5 μM TPBM inhibited binding of TPBM to the flcERE by ~60%. Data were plotted with the change in anisotropy for binding of $E_2$-ERα to the flcERE in the absence of small molecule inhibitors set to 100% (actual anisotropy: flcERE: 44 mA units; $E_2$-ERα-flcERE: 81 mA units). Data are the average ± SEM of 4 experiments. The difference between 5 μM TPSF and the control (no inhibitor) was not significant ($p>0.05$). The data for 5 μM TPBM was significantly different from both the control and from 5 μM TPSF ($p<0.01$ using Student's t test) *B*. TPSF decreases ERα levels. MCF-7 cells were cultured in 5% CD-calf serum for at least 2 days and maintained in +/- $E_2$ and the indicated concentrations of TPSF or TPBM for 24 h. Lysates were then analyzed for ERα by Western blot using 8 μg protein/lane,

with actin as internal standard. Data are from the 3 independent western blots and are presented as mean ± SEM. Quantitation of ERα and actin was by PhosphorImager analysis. The value for ERα/actin in the absence of $E_2$ was set equal to 1. C. T47D cells were maintained in the absence or presence of $E_2$ and the indicated concentrations of TPSF, harvested, and analyzed by western blot as described for panel *B*.

Experimental Data in Figure 2.7 B, C: C Mao

**Figure 2.8**

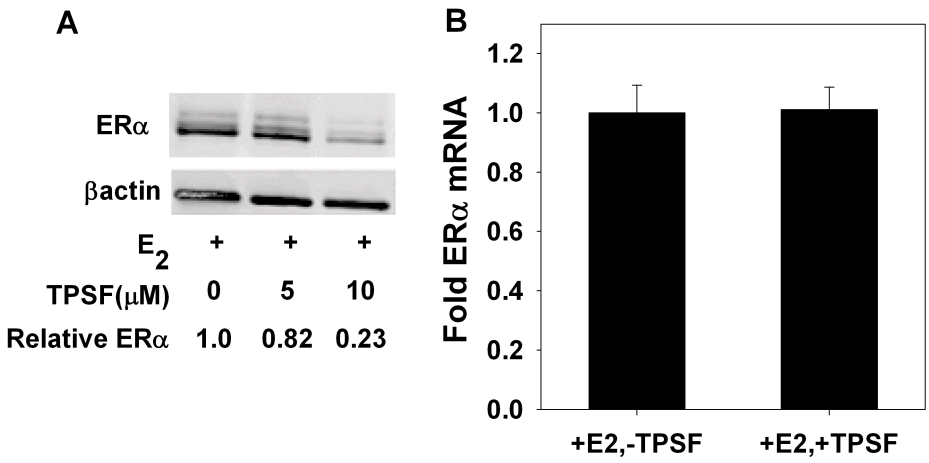



FIGURE 2.8. **TPSF does not alter the level of ERα mRNA.** *A*. Western blot of HeLa-ER cells extract. HeLa cells stably transfected to express functional wild-type ERα (76) were maintained in MEM+10% FBS. Four days before the cells were plated in 6-well plates at 50,000 cells/well in MEM + 10% 1X CD-FBS. The medium was changed after 2 days and on day 4 replaced with fresh medium containing 10 nM $E_2$ in DMSO, or DMSO and the indicated concentration of TPSF. After 24 h, the cells were harvested and extracts prepared as described in Material and Methods. *B*. Effect of TPSF on ERα mRNA levels in MCF-7 cells. Cells were maintained 4 days in MEM + 5% 1X CD-FBS as described in Material and Methods. Then cells were then maintained for 24h in medium containing 10 nM $E_2$ in DMSO or DMSO with or without 10 μM TPSF and ER mRNA levels determined by qRT-PCR as described in Material and Methods. Data were the average of 3 experiments ± SEM.

Experimental Data in Figure 2.8: C Mao

**Figure 2.9**

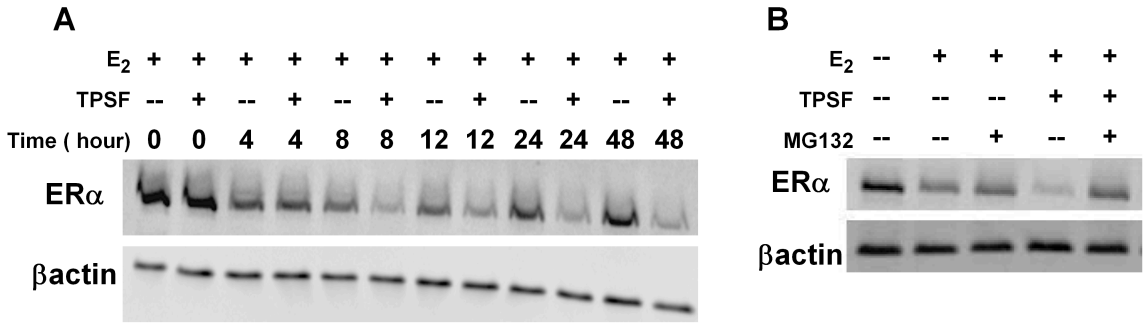



FIGURE 2.9. **The proteasome inhibitor MG132 blocks degradation of ERα by TPSF.** *A*. Time course of the effect of TPSF on ER levels. MCF-7 cells were plated as described in Materials and Methods. After 4 days in MEM + 5% 1X CD-FBS, the medium was replaced with medium containing 10 nM $E_2$ with or without 10 μM TPSF. Cells were harvested at the indicated times and extracts prepared and ERα protein levels determined by western blot as described in Materials and Methods. *B*. MG132 reverses the down-regulation of ER by TPSF. Cells were treated as in panel A and maintained for 24 h in medium containing 10 nM $E_2$ with or without 10 μM and 10 μM MG132. Preparation of cell extracts and western blotting were as described in Materials and Methods.

Experimental Data in Figure 2.9: C Mao

**Figure 2.10**

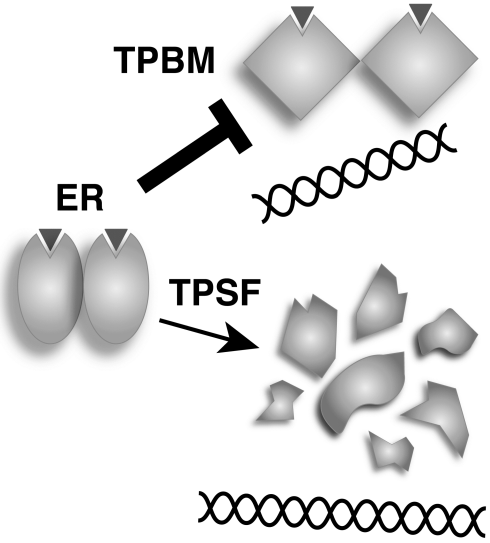
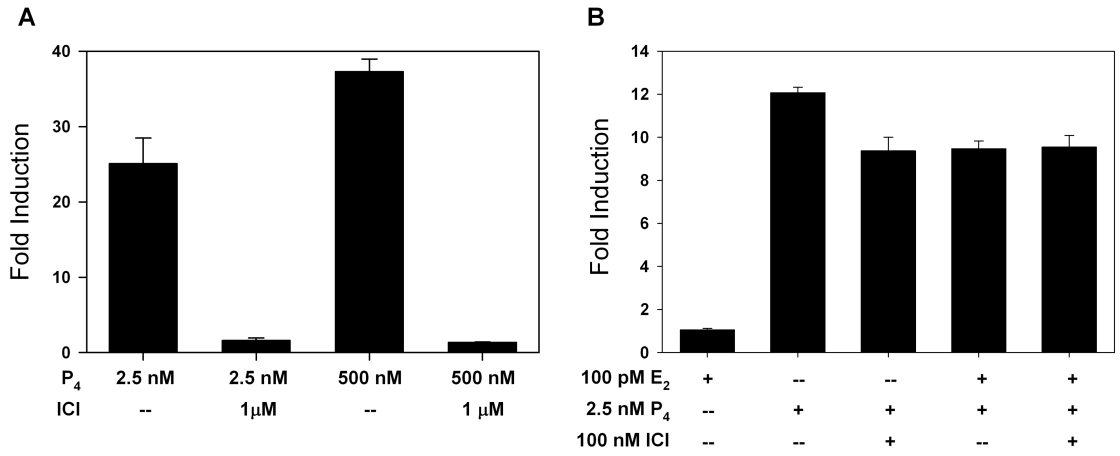



FIGURE 2.10.

**Schematic representation of the different modes of action of TPSF and TPBM.**

**SUPPLEMENTAL FIGURES**

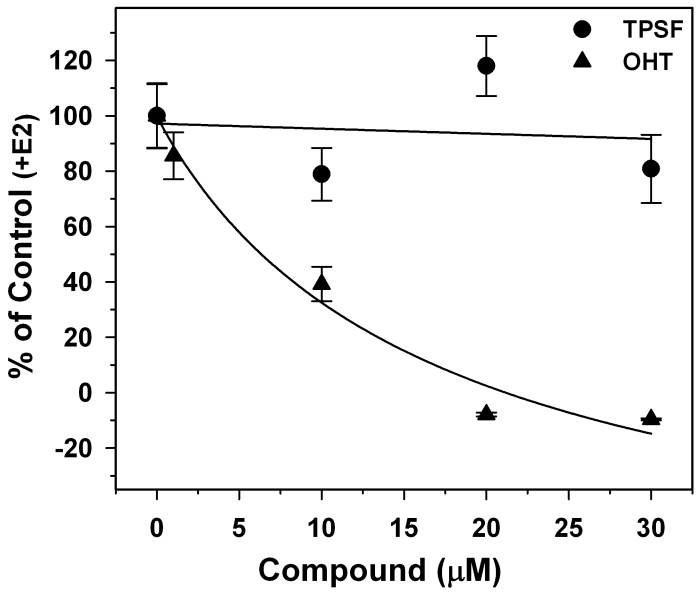
**Supplemental Figure 2S.1**




SUPPLEMENTAL FIGURE 2S.1. **The ER inhibitor ICI 182,780, decreases PR-mediated gene expression in T47D cells**. In panel A before plating the cells were maintained in full serum (5% FBS) that contains some estrogen. In panel *B* the cells were depleted of estrogen by 4 days in medium containing 5%1X CD-FBS. A. 24 h after plating 2.5 nM or 500 nM progesterone ($P_4$) was added with or without 1 μM ICI 182,780. *B.* After 24 h cells were maintained in medium with or without 5 nM progesterone ($P_4$) and 1 μM ICI 182,780. Alkaline phosphatase assay. After 24 h in the indicated treatment, the cells were washed once with PBS, lysed in 100 μl of buffer (20 mM potassium phosphate, pH 7.8, 5 mM $MgCl_2$, 0.5% Triton X-100, frozen. Lysate was stored at –70˚. 5 μl of supernatant was removed and assayed in a 96 well plate in 25 μl of assay buffer (100 mM diethanolamine, pH 9.5, 1 mM $MgCl_2$, 0.4 mM CSPD (substrate, Applied Biosystems/Tropix) and 1X Emerald ll enhancer (Applied Biosystems/Tropix). After 1 h at room temp., luminescence in the visible (emission max. 542 nM) was read for 1 sec. Data were the average of 3 experiments ± SEM.

Experimental Data in Supplemental Figure 2S.1: IO Aninye

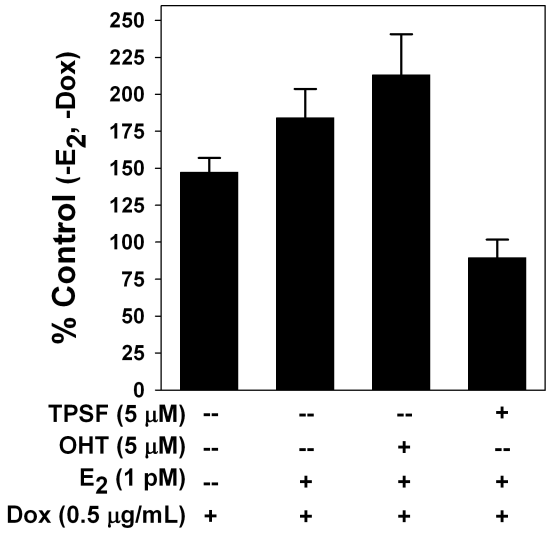
**Supplemental Figure 2S.2**




SUPPLEMENTAL FIGURE 2S.2. **TPSF does not exhibit nonspecific toxic effects in ERα negative MDA-MB-231 cells.** Cell maintenance, growth and plating were as described in the Fig 2.4 legend. The indicated concentrations of OHT (filled triangles) and TPSF (filled circles) and 1 pM $E_2$ were added and cell number was determined using MTS and a standard curve. Cell number in the absence of TPSF or OHT was set as 100%. Data were the average of 3 experiments ± SEM.

Experimental Data in Supplemental Figure 2S.2: NM Kretzer

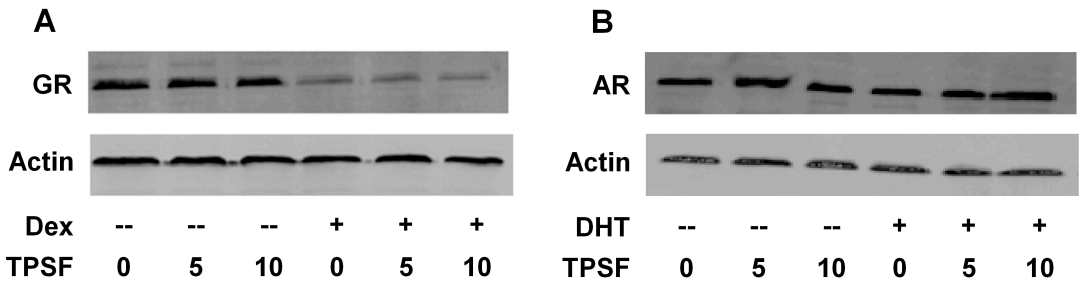
**Supplemental Figure 2S.3**




SUPPLEMENTAL FIGURE 2S.3. **TPSF but not OHT inhibits $E_2$-ERα-dependent growth of MCF7ERαHA cells.** MCF7ERαHA cells were maintained for 4 days in 6x CD-FBS (22,37), seeded into 12 well plates with or without 0.5 μg/ml Dox , 1 pM $E_2$, 5 μM OHT and 5 μM TPSF, grown for 4 days and assayed using MTS and a standard curve for cell number. Cells in ethanol and DMSO vehicle alone were set to 100%. Data represent the average of 3 experiments $\pm$ SEM. The difference between the cells incubated with TPSF + $E_2$ + Dox and cells in OHT + $E_2$ + DOX was significant ($P < 0.05$ using Student's t test).

Experimental Data in Supplemental Figure 2S.3: IO Aninye

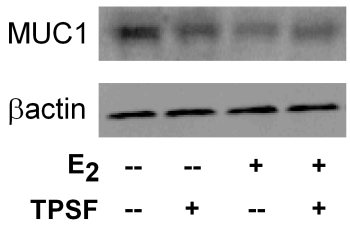
**Supplemental Figure 2S.4**




SUPPLEMENTAL FIGURE 2S.4. **TPSF has little or no effect on levels of AR and GR.** To test the selectivity of TPSF for down-regulation of ERα, we evaluated the effect of 5 μM and 10 μM TPSF on the levels of AR and GR. The cell lines used and cell growth conditions were those employed in the dose-response studies evaluating effects of TPSF on gene expression (Fig 2D). Cells were maintained in medium containing or lacking dexamethasone (panel A) or DHT (panel B) and 0, 5 or 10 μM TPSF for 24 hours. Extracts were prepared and analyzed on separate gels for AR and GR levels by western blotting. Total protein (12 μg for AR and 40 μg for GR) was fractionated on 10% polyacrylamide gels and AR and GR levels determined by western blot. The antibody used to detect AR was AR441 monoclonal antibody used at 1:1000 dilution (NeoMarkers, Freemont, CA). The GR antibody was MA1-510 monoclonal antibody (Thermo Scientific, Rockford, IL) used at 1:1000 dilution. Data is representative of at least 2 gels for each sample.

Experimental Data in Supplemental Figure 2S.4: IO Aninye, MC Cherian and C Mao

**Supplemental Figure 2S.5**




SUPPLEMENTAL FIGURE 2S.5. **TPSF does not down-regulate the level of Muc1.** MCF-7 cells were maintained and plated as described in Materials and Methods. After 4 days in MEM + 5% 1X CD-FBS, the medium was replaced with medium with or without 10 nM $E_2$ and with or without 10 μM TPSF. Cells were harvested at 24 h extracts prepared and Muc1 detected using Muc1 specific antibody (60). The actin samples were run on a separate gel.
Experimental Data in Supplemental Figure 2S.5: C Mao

# CHAPTER 3
# HIGH-THROUGHPUT SCREENING FOR IDENTIFYING INHIBITORS OF ANDROGEN RECEPTOR ACTION

Prostate cancer (PCa) is one of the leading causes of male mortality in the United States with more than 240,000 diagnosed cases predicted for 2012 alone (1). Androgens working through androgen receptor (AR) are fundamental to the growth and proliferation of prostate cancers. AR is the best-studied and most targeted biomarker of this type of cancer. Early stage tumors undergo remission with surgical or chemical castration, sometimes combined with radiation. The primary line of defense upon diagnosis of prostate cancer includes treatments that reduce circulating levels of androgens (androgen ablation) such that the stimulation of proliferation by androgen-bound AR is inhibited and the tumor regresses. Often, androgen deprivation therapy includes the use of antiandrogens (bicalutamide, hydroxyflutamide, nilutamide) to directly antagonize AR transactivation. But about 25% of the patients diagnosed with PCa develop 'resistance' to current treatments that aim at androgen ablation. These tumors called castration-recurrent prostate cancer (CRPC) are highly malignant, able to grow in low circulating androgens or even anti-androgens. Treatment options for these aggressive tumors are limited. Androgen ablation is not a *cure* for prostate cancer, but instead has an anti-proliferatory effect on advanced prostate cancer. Eventually a fatal form of cancer will remerge in the castrate environment, in which AR is reactivated by poorly understood molecular mechanisms that enable the tumor to be castration-recurrent. However, androgen receptor continues to play a central role in CRPC progression.

My goal was to use high-throughput screening (HTS) to identify new small molecule inhibitors of AR action that have the potential for therapeutic use in CRPC. As AR in the castration-recurrent phenotype is unaffected by classical ligand antagonists, I wanted these inhibitors to preferentially act outside of the ligand-binding domain (LBD) of AR. With this idea, a moderate-scale screen (~12,000 small molecules) was set up using the fluorescence anisotropy microplate assay (FAMA) developed previously in our lab (2, 3). Small molecules may inhibit the macromolecular surfaces involved in DNA binding of steroid receptors, by either physically occupying the contact interface, or by altering protein conformation and are detectable by a change in fluorescence anisotropy. Later, a higher-throughput screening was set up and implemented using a stable HeLa cell line expressing androgen-induced, AR-mediated luciferase

reporter. This chapter details the various screening methodologies developed for the identification of small molecule inhibitors of AR action reported in this thesis.

## (I) FAMA FOR IDENTIFYING INHIBITORS OF AR BINDING TO DNA

Previously it was thought that because protein-DNA and most protein-protein interfaces are large, they were poor targets for HTS to identify small molecule inhibitors. But we had considerable success in using FAMA to identify inhibitors of estrogen receptor binding to estrogen responsive element constructs (4). These inhibitors were also able to disrupt these complexes and interrupt transactivation of target genes in MCF-7 as seen in RT-PCR and chromatin immunoprecipitation assays. A similar approach was used to identify AR inhibitors.

Briefly, small biomolecules such as short DNA fragments tumble rapidly in solution due to their increased entropy. If these biomolecules are labeled with a fluorescent dye, a beam of polarized light passing through this solution will be mostly depolarized and light is detected at both the parallel and perpendicular detectors. When a second biomolecule known to interact with the first is introduced into this solution, the ensuing complex rotates more slowly in solution due to its conformational change and the light emitted is more polarized. An inhibitor of this interaction would in principle result in less polarized (more depolarized) emissions, without exhibiting any intrinsic fluorescence (Fig 3.1). We rule out ligand-exchange in these assays, as the amount of DHT added in the reaction buffer is substantial, DHT is the most effective ligand known for AR. Moreover, the AR protein was purified with DHT.

I used full-length flag-tagged AR expressed and purified from baculovirus cultures by our collaborator Dr. Elizabeth M. Wilson (UNC, Chapel Hill). I successfully applied FAMA to the binding of AR to three fluorescein-labeled palindromic androgen response elements (ARE), the fl-cARE, fl-c3ARE and fl-PRE/GRE. The assay is done in a total volume of 20 µl in 96-well micro-plates. Increasing concentrations of AR are incubated with 1 nM of the fluorescein-labeled probe, for 10-15 minutes at room temperature in the dark prior to measuring the polarization/anisotropy. The assay binding buffer contains: 20 mM Tris-Cl pH 7.5, 100 mM KCl, 2 mM dithiothreitol (DTT), 10% glycerol, 0.25 µg/µl bovine serum albumin (BSA), 0.2 mM EDTA and 10 ng of non-specific DNA, poly (dI:dC). These conditions were shown to abolish non-specific binding. The dilution buffer for AR was similar, with 5 µM $ZnCl_2$ for stabilizing the zinc-finger motifs within the AR DNA-binding domain (DBD) and 1 µM dihydrotestosterone (DHT) to keep the receptor ligand-bound and hence stable for longer periods of time (5). The

fluorescence anisotropy is measured as a 'change in anisotropy' in milli-anisotropy (mA) units, representing the difference between anisotropy values measured at each concentration of AR and values measured in the wells with free probe. An approximate $K_D$ (dissociation constant) was determined as the receptor concentration at which 50% of the probe was bound. AR bound to the three probes with an apparent $K_D$ ~25 nM in 100 mM salt, which is thought to be a physiologically relevant salt concentration (Fig. 3.2).

I used 1 nM fl-cARE for the screen with 45 nM AR, which results in at least 80% of maximal binding. The test compounds in each plate were added at a final concentration of 2.5 μM using a 100 nl pin-spotter (VP Scientific) and incubated for 15 minutes before measuring the fluorescence anisotropy using the BMG Pherastar (excitation wavelength 495 nm, emission wavelength 520 nm). Z'-factor of the screen was calculated to be 0.8 using the formula given below, which is characteristic of robust assays whose precision and dynamic range is suitable for HTS development.

$$\textbf{Z'-factor} = 1 - \left[ \frac{3 * (\textbf{STD}_{\textbf{sample}} + \textbf{STD}_{\textbf{P}})}{|\textbf{Mean}_{\textbf{sample}} - \textbf{Mean}_{\textbf{P}}|} \right]$$

where $\textbf{STD}_{\textbf{sample}}$ and $\textbf{Mean}_{\textbf{sample}}$ are the sample mean and standard deviation from the mean, $\textbf{STD}_{\textbf{P}}$ and $\textbf{Mean}_{\textbf{P}}$ are the mean and standard deviation of the negative control wells on the plate.

The actual primary screen of ~12,000 small molecules was relatively small and was performed within a week, with help from Dr. Chengjian Mao. Fig 3.3 represents a typical plate from the screen. % Inhibition of each compound was calculated as

$$\textbf{\% Inhibition} = 1 - \left[ \frac{(\mathbf{x}_{\textbf{well}} - \ddot{\mathbf{X}}_{\textbf{P}})}{(\ddot{\mathbf{X}}_{\textbf{P}})} \right]$$

where $\mathbf{x}_{\textbf{well}}$ is the change in anisotropy measured for each compound in a well and $\ddot{\mathbf{X}}_{\textbf{P}}$ is the change in anisotropy measured in the control wells (DMSO) containing AR: flcARE.

The most dramatic increases or decreases in anisotropy were observed in wells with compounds that exhibit intrinsic fluorescence. These were eliminated on the basis of increased deviation of % fluorescence intensity when compared to control wells containing DMSO. On an average 3-4 inhibitors were identified per plate of small molecules. The ~200 molecules identified as inhibitors in the first round of screening were used in the secondary screening

assays including dose-response curves. Specificity of the small molecules for AR was tested by determining the effect on estrogen and progesterone receptors binding to their respective response elements in FAMA. Molecules that showed high potency and specificity were then utilized in cell-based androgen-mediated luciferase assays and in androgen-dependent prostate cancer cell proliferation assays.

To our great disappointment, most specific inhibitors of AR:ARE in FAMA when tested in cell-based assays showed no or low inhibition of androgen-induced AR-mediated luciferase activity. Only one inhibitor showed any inhibition of AR in a dose-responsive manner and translated well into a cell-based assay system. The structure of this small molecule, #4457, is illustrated in Fig 3.4A and it specifically and potently inhibited AR-ARE interaction (Fig 3.4B). #4457showed some dose-dependent inhibition of androgen-induced AR-mediated luciferase reporter in HeLa cells (Fig 3.4C). But a closer look at the compound revealed that it is a TNT derivative and thus challenging for chemists to synthesize more of the compound or make analogues for structure-activity relationship study. We decided not to pursue this compound, instead planned to perform an unbiased cell-based screen.

I also investigated ~180 small molecules that were structurally similar to the ER inhibitor identified using FAMA (TPBM, (4)) and conducted structure-activity relationship studies with all the steroid receptor activated luciferase reporter systems. This was performed in hopes of identifying potent and specific small molecule inhibitors that may inhibit AR action. Two of these inhibitors, #95869 and #95899, performed with reasonable specificity and potency in cell-based luciferase reporter assays, induced using 10 nM DHT (Fig 3.5A). But in radio-ligand competition assays, these compounds were found to be competitive antagonists that worked less well than bicalutamide (Fig 3.5B). Since the main purpose of the screen was to identify potent non-competitive inhibitors of AR action for potential use in a castrate-recurrent environment, the inhibitors identified so far were underwhelming and hence not pursued any further.

There are two basic approaches to HTS. A small library can be screened and candidate molecules subject to further study by combinatorial synthesis of related compounds and further evaluation and testing. This is what I had hoped to do with the moderate-scale fluorescence anisotropy screen, but the results were not ideal. Alternatively, a much larger library can be screened with the hope that individual hits will be identified with better potency, efficacy and specificity. So I decided to use this second approach by utilizing the ~160,000 small molecules in the chemical libraries at the HTS facility on this campus. I hoped that this type of large screen

in an AR-regulated luciferase-reporter system is more likely to generate strong lead compounds capable of crossing the cell membrane for inhibition of AR action.

## (II) CELL-BASED HIGH-THROUGHPUT SCREENING FOR IDENTIFYING INHIBITORS OF ANDROGEN RECEPTOR ACTION

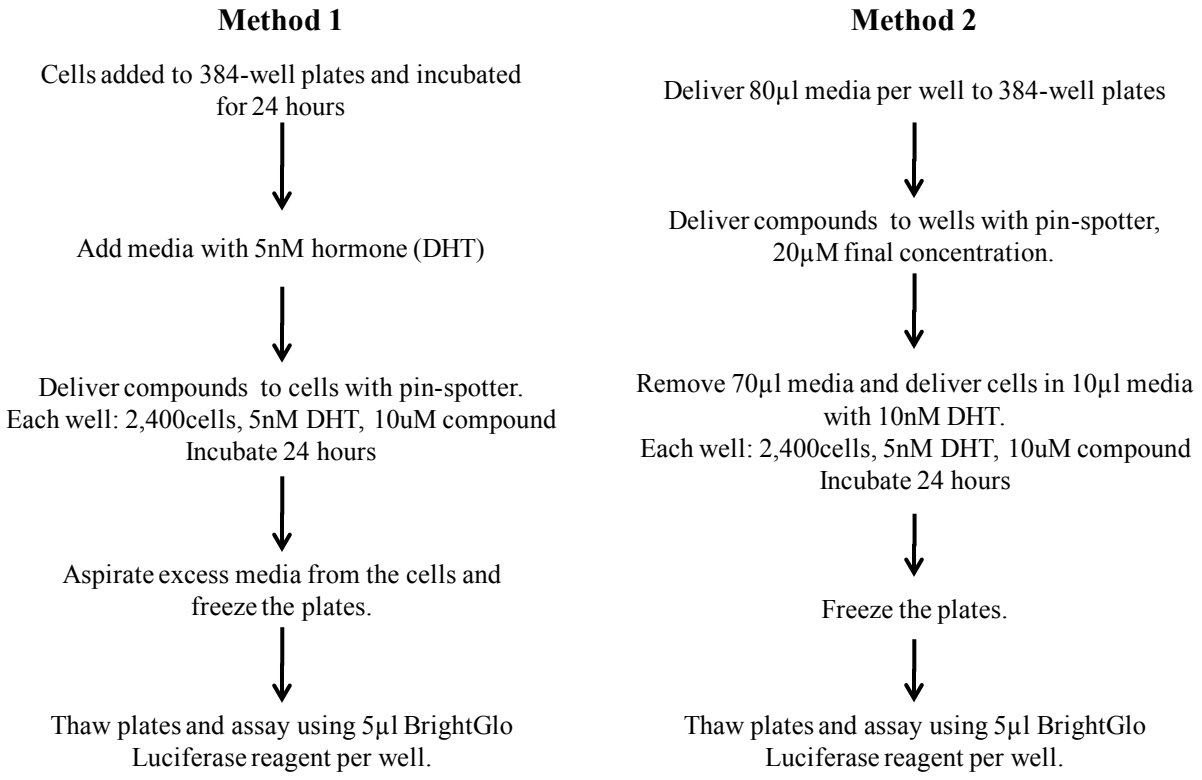
Cell-based assays combine the biological complexity of live cell responses with the scalability and process adaptation of HTS. The application of cell-based assays in HTS is an inherently challenging operation. The large biological complexity and high degree of crosstalk between integrated signaling pathways predispose cell-based assays to more false positives than biochemical assays (6). The high biological complexity also increases potential sources of noise and variability that can lead to deterioration of assay performance to levels unacceptable for HTS. These factors include variations in cell plating, differential cell growth and response, uneven response across plates, plate-to-plate variability, and edge effects.

The feasibility of performing a cell-based high-throughput screen was tested extensively before set-up. To overcome well-to-well variability that could arise if aspiration steps are performed from well containing cells, we compared two different set-ups for screening in 384-well plates.

In testing a large number of compounds for AR-mediated transactivation and for toxicity of the hits, it would be simplest to add the cells and the small molecules to the wells at the same time. The more traditional alternative we previously used involved plating the cells, waiting for them to attach and grow for 24 hours, and then adding the compound. But this results in significant additional labor and poses evaporation problems because of the very small volume of medium in the wells. Method 1 involves plating the cells one day prior to compound addition and aspirating the excess media from the cells after 24 hours of incubation with small molecules. If aspiration caused cells to dislodge from the bottom of wells, we felt that this step could increase the number of false positives.

In method 2, cells are delivered after the compound and no medium aspirations are required after cell addition, thus reducing the chances of variability due to cell number. A hit map of results from a test plate comparing the two above methods is illustrated in Fig 3.6. Inhibition of transactivation and toxicity of the small molecules were similar when the compounds were added along with the cells and when the compounds were added after the cells had had a day to attach

and recover. Thus we chose method 2 for large-scale screening of the ~160,000 small molecules in the library.




Careful consideration was given to way to reduce '*edge effect*'. Thermal gradients are greatest in the edge wells of plates, and they result in uneven distribution of the cells on the well bottoms. The uneven cell distribution in turn affects cell adhesion and morphology, and response to test compounds. After adding the cells, the plates were arranged in the 37°C, 5% $CO_2$ incubator in single files across each shelf (rather than stacks) and care was taken not to open the incubators until the end of the 24 hour period. This seems to help in maintaining equal volumes in wells across the plate. Due to the way the chemical libraries are set-up, the edge columns and rows of plates had to be evaluated in the assay.

## MATERIALS AND METHODS

***Screening libraries-*** The main library we screened was the ChemBridge MicroFormat™ library, which was pre-plated in 10 mM stocks in DMSO. We also included two smaller libraries,

the Marvel library[1], containing ~9,700 small molecules (4, 7, 8) and the HTSF library, an in-house compound library.

***Cell lines-*** Our collaborators at the University of North Carolina, Chapel Hill, developed two stable cell lines in HeLa cells for this study. HeLa-AR1C-PSA-ARE-Luc-A6 (HeLaA6) cells (9) that stably express androgen receptor (AR) and a prostate-specific antigen (PSA)-Luc reporter were maintained in MEM supplemented with 10 mM HEPES, 1% penicillin-streptomycin (P/S), 10% FBS, and under selection with 0.2 mg/ml hygromycin B and 0.5 mg/ml G418. HeLaA6 cells express considerably more AR protein when compared to prostate cancer cell lines like LNCaP or LAPC4. (Fig 3.7) In this regard, they behave more like castration-resistant prostate cancers (CRPCs). HeLa-AR3A-PSA-ARE-Luc-13 (HeLa13) cells that stably express AR and a PSA-Luc reporter were maintained in MEM supplemented with 10 mM HEPES, 1% penicillin- streptomycin (P/S), 10% FBS, and under selection with 0.1 mg/ml hygromycin B and 0.5 mg/ml G418. HeLa13 cells express AR protein levels similar to LNCaP cells (Fig 3.7).

T47D-KBluc breast cancer cells expressing an $(ERE)_3$-luciferase reporter gene (10) were maintained in RPMI 1640 containing 10% FBS. T47D/(A1-2) cell line that stably express the glucocorticoid receptor (GR) and contain a mouse mammary tumor virus (MMTV)-luciferase reporter (11) were maintained in MEM, 5% FBS and 0.2 mg/ml geneticin (G418).

LNCaP cells were maintained in phenol-red free RPMI 1640 with 10% fetal bovine serum (FBS; Atlanta Biological, GA). Cells were transferred to RPMI 1640 containing 5% CD (charcoal-dextran stripped) FBS at least 3 days prior to plating for an experiment.

***Reporter Gene Assays***- For the primary screen 2,400 HeLaA6 cells were added per well of 384-well plates and treated with 5 nM DHT and 7.1 μM compound. For the secondary screen, we seeded 2,400 HeLaA6 cells or 4,500 HeLa13 cells per well of 384-well plates. At least 3 days before each experiment, cells were switched to medium containing CD-treated FBS as described above. For the ER luciferase assays, T47D-KBluc cells were plated at 8,000 cells/well and induced with 5 nM estradiol ($E_2$). Similarly, T47DA/1-2 cells were used to assay the effects of compounds on the glucocorticoid receptor (GR). After 24 hours of incubation with the compound and respective hormone or other ligands, cells were lysed partially by freeze-thaw and assayed using 5 μl per well of BrightGlo reagent (Promega, Madison WI). Unless otherwise

[1] The Marvel library was developed at the University of Illinois by Dr. K. Putt and Professor P.J. Hergenrother.

stated, total DMSO (vehicle) concentration in the assays was maintained between 0.1-0.5 percent.

***Cell Growth and Toxicity Assays***- At least 3 days prior to plating, LNCaP cells were maintained in medium containing 5% CD-FBS while DU145 cells were maintained in their growth medium. For all cell lines, 2,000 cells/well was added to 96-well plates and allowed to attach for 1 day. Cells were added with treatment medium containing the indicated concentrations of hormone and small molecule and incubated for 3-4 days. Cell viability (MTS) assays were carried out using the CellTiter 96 Aqueous One Solution cell proliferation assay (Promega).

***Luciferase-enzyme activity assay-*** To test whether small molecule leads obtained from the AR screen were inhibitors of luciferase enzyme, we performed assays using QuantiLum recombinant luciferase protein (Promega). For each 10 µl assay in a 384-well plate, 0.05 ng of luciferase protein in 5 µl buffer was incubated with 10 µM small molecule. After 10 minutes, 5 µl BrightGlo was added and luciferase activity was measured.

**PRIMARY HIGH-THROUGHPUT SCREENING**

***Screening Method-*** The primary HTS was conducted at the High Throughput Screening Facility (HTSF) using robotic equipment. Prior to each experimental batch, HeLaA6 cells were grown in multiple flasks in MEM + 10% FBS. The day before the plating the cells, 70 µl of MEM + P/S was added to each well of 30-60 white 384-well plates under sterile conditions with a Matrix WellMate 8-channel dispenser. Using a pin-spotter (V&P Scientific Inc.) mounted on a Matrix Platemate-Plus, 100 nl of 10 mM compound in DMSO from each plate of the library was transferred to the assay plates and mixed. DMSO was added to the positive and negative control wells (2x16 wells). 60 µl was aspirated out automatically using Matrix Platemate-Plus, such that 10 µl of the medium containing 14.2 µM compound was left behind in each well. Cells were trypsinized into MEM+ 10% FBS, 10 nM DHT was added and plated at 2,400 cells/well in 10 µl volume in a sterile environment. Each well thus contained 2,400 cells, 5 nM DHT and 7.1 µM compound in 20 µl of MEM + 5% FBS. The cells were incubated at 37°C in 5% $CO_2$ for 24 hours and then frozen at -20° C for at least 6 hours before reading the luciferase activity. We believe that the freeze-thaw cycle facilitates cell lysis. Plates were brought to room temperature along with the reconstituted BrightGlo reagent (Promega) and 5 µl of BrightGlo was added per well. This mix was incubated 15 minutes at room temperature for increasing cell lysis and

release of luciferase enzyme, and then luminescence was read the Analyst HT (Molecular Devices).

***Analysis-*** Data from the screen was analyzed using Microsoft Excel. The top 50 and bottom 50 luciferase values of every plate was discounted in the calculation of the average plate value so that the average used in the calculation of % activity of each well is not biased towards activators or inhibitors. % Activity was calculated using the formula

$$\% \text{ Luciferase Activity} = \frac{(Luc_{well} - Luc_{plate\ average})}{Luc_{plate\ average}} \times 100$$

where $Luc_{well}$ is the optical units measured from any given well, $Luc_{plate\ average}$ is the average calculated discounting the top 50 and bottom 50 values. The effect of each compound on luciferase activity was determined by comparison to the positive and negative control wells of each plate. Compounds showing > 50% luciferase inhibition were selected for further study in the secondary screen. Approximately 3000 compounds were selected. A schematic of the various steps leading to the identification and characterization of '*lead*' AR inhibitors are illustrated as a flowchart in Fig 3.8.

Note: Although I used what seems like an arbitrary value of >50% inhibition as a metric to peg a compound well as a 'hit', it was > 3 times the standard deviation of the controls. Later these values were crosschecked using the strictly standardized median deviation (SSMD*) method, which scores inhibitors from a range of poor (scores = 0, corresponding to no effect) to very strong (score ≤ -5, corresponding to greater than 99 percent confidence interval). It was determined that all the hits identified from any plate corresponded to scores between -1.5 and -3, which corresponds to 87-99 percent confidence intervals respectively (6).

## SECONDARY HIGH-THROUGHPUT SCREENING

**A.** To verify the ~3,000 primary hits and test them for potency, efficacy, specificity and toxicity we needed to perform a large number of assays. A secondary screen library was created by manually picking 1 µl of each hit from a 1 mM copy of the original 10 mM library (a process known as *cherry picking*). This new library was then diluted in Hanks Balanced Salt Solution (HBSS) and MEM (no serum) just before use. A final concentration of 8 µM of each compound was used in all assays. Secondary screening involved repeating the primary screen in HeLaA6 cells as well as other stringent tests.

**B.** We also selected 160 compounds from the primary screening to make a *second* secondary screening library, by manually picking 1 µl from the original 10 mM library. This was due to the inconsistencies we noticed after the first round of secondary screening and realization that data from assays performed using the 1 mM copy library was significantly different from those obtained from the original 10 mM compound library. Multiple freeze-thaw cycles had apparently further diluted the 1 mM library (as water is absorbed by DMSO at ambient temperatures).

***Screening method-*** Secondary screening assays were performed in much the same way as primary screening in 384-well plates. Luciferase inhibition by compounds acting through AR in HeLaA6, HeLa13 and through ERα in T47D-KBluc cells were measured in duplicate. Toxic effects of the chosen leads, if any, on the HeLa cells were determined using MTS reagent. The data from all the above assays was used to further shortlist AR inhibitor leads.

***Analysis-*** The effect of each compound on luciferase activity was determined by comparison to the positive and negative control wells of each plate. Compounds showing >65% inhibition of luciferase activity in the AR containing cell lines, which were also >75% viable and had minimal inhibitory effects on ER, were selected for further study. Approximately 120 small molecules were shortlisted at this stage for further study.

## TERTIARY HIGH-THROUGHPUT SCREENING

We tested the selected 280 (120+160) compounds in two batches at various doses on the HeLa13 cells and determined the extent of inhibition of luciferase activity in 96-well plates. This was compared to inhibition of each compound on ER-mediated and GR-mediated luciferase activity in T47D cells. The growth inhibitory effects of all the compounds were tested in triplicate assays in AR-dependent LNCaP cells. Finally, 37 small molecules from the first batch and 12 small molecules from the second batch were selected for purchase from ChemBridge or acquired from the NIH (Developmental Therapeutics Program). Due to cost considerations, the number of small molecules purchased was kept to a minimum.

## LEAD DEVELOPMENT

The 37 compounds obtained in the first batch were assayed for inhibition of AR-dependent growth in LNCaP cells, luciferase expression, and for AR-independent toxic effects in DU145 cells. Table 3.1 represents the data obtained from testing 10 µM of each compound in the various assays. Highlighted in red are the reasons for eliminating that particular compound. Only two

compounds (*:AR19 and AR33) showed the desirable qualities of reducing AR-dependent cell growth and transactivation, without intrinsically effecting luciferase enzyme activity. From the dozen small molecules in the second batch purchased from ChemBridge, two compounds (AR45 and AR54) inhibited AR-dependent transactivation and proliferation with reasonable $IC_{50}$s. We evaluated a total of 4 lead compounds, AR19 (CPIC), AR33, AR45 and AR54. AR33 and AR45 were later dropped from mechanistic studies due to their toxicity profile in AR-independent cell lines. Extensive mechanism of action studies were performed for AR19 (CPIC) detailed in Chapters 4 and 5, and AR54 described in Chapter 6.

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## TABLES & FIGURES

**Table 3.1: Analysis of the effects of 10 µM of the compounds purchased from ChemBridge.**

| Compound (10µM) | DU145 % Viability | LNCaP % Growth | HeLa13 % Luc Activity | Luciferase Enzyme % Activity |
|---|---|---|---|---|
| DMSO | 100.0 | 100.0 | 100.0 | 100.0 |
| AR1 | 122.9 | 70.7 | 45.3 | 94.7 |
| AR2 | 71.7 | 55.4 | 8.4 | 41.1 |
| AR3 | 107.0 | 63.2 | 10.8 | 17.3 |
| AR4 | 85.6 | 78.7 | 15.3 | 13.0 |
| AR5 | 76.7 | 83.5 | 17.7 | 102.2 |
| AR6 | 104.4 | 70.5 | 178.5 | 13.7 |
| AR7 | 145.3 | 52.4 | 41.7 | 106.0 |
| AR8 | 147.4 | 64.4 | 30.4 | 19.7 |
| AR9 | 113.0 | 64.9 | 14.2 | 12.6 |
| AR10 | 31.0 | 36.1 | 30.6 | 102.9 |
| AR11 | 109.7 | 69.7 | 203.1 | 97.9 |
| AR 12 | 95.1 | 87.5 | | 52.5 |
| AR 13 | 2.0 | 12.8 | 64.1 | 56.1 |
| AR 14 | 89.4 | 70.0 | 3.7 | 12.8 |
| AR 15 | 93.9 | 69.9 | 50.9 | 18.0 |
| AR 16 | 6.1 | 5.2 | 11.6 | 89.6 |
| AR 17 | 100.0 | 108.0 | | 22.0 |
| AR 18 | 85.5 | 84.8 | | 90.9 |
| AR 19* | 62.9 | 42.4 | 2.8 | 99.8 |
| AR 20 | 93.3 | 90.8 | 103.6 | 33.3 |
| AR 21 | 85.5 | 62.1 | 86.0 | 102.7 |
| AR 22 | 71.7 | 104.3 | 12.6 | 105.6 |
| AR 23 | 81.7 | 77.3 | 2.3 | 100.8 |
| AR 24 | 96.0 | 126.6 | 46.0 | 27.9 |
| AR 25 | 97.8 | | 106.7 | 99.2 |
| AR 26 | 93.6 | | 170.2 | 95.8 |
| AR 27 | 79.7 | | 91.6 | 95.8 |
| AR 28 | 93.0 | | 104.1 | 96.4 |
| AR 29 | 57.6 | | 35.1 | 92.0 |
| AR 30 | 90.0 | | 106.3 | 88.3 |
| AR 31 | 92.5 | | 78.2 | 92.3 |
| AR 32 | 79.1 | | 92.2 | 94.3 |
| AR 33* | 82.9 | 1.9 | 20.5 | 98.8 |
| AR 34 | 120.1 | | 75.8 | 99.9 |
| AR 35 | 15.1 | | 11.8 | 105.2 |
| AR 36 | 12.6 | | 5.4 | 105.8 |
| AR 37 | 10.0 | | 9.8 | 101.3 |
| Bic | | 35.0 | 24.0 | |

* Represents lead inhibitors

**Figure 3.1**

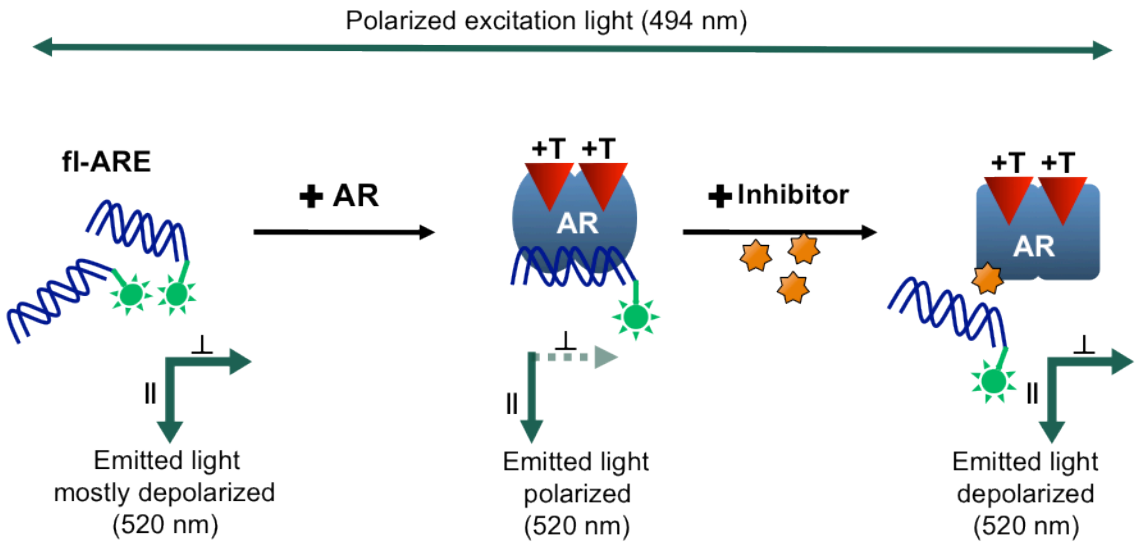



**FIGURE 3.1. Schematic representation of FAMA analysis of AR:DNA and AR:DNA-inhibitor interactions.**

When excited with polarized light of 494 nm, the fluorescein-labeled androgen response elements, fl-ARE, tumbles rapidly in solution and the emitted light is largely depolarized to the parallel and perpendicular detectors (520 nm). Binding of DHT (T)-bound AR to the fl-ARE results in a much larger, less mobile complex. Therefore, the emitted light is mostly polarized. Binding of small molecule inhibitors that disrupt the AR:fl-ARE complex reverses this polarization, resulting in emitted light that is more depolarized and a decrease in fluorescence anisotropy.

**Figure 3.2**

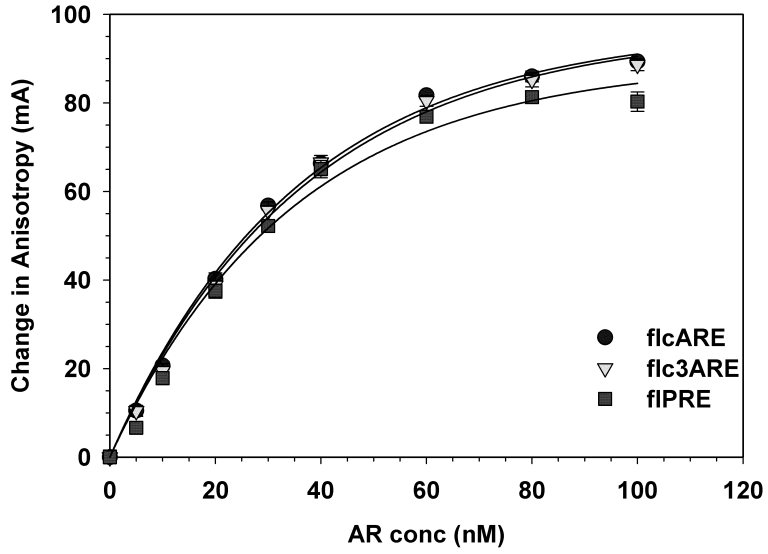



FIGURE 3.2. **FAMA analysis of the binding of AR to different hormone-response elements (HREs).** Increasing amounts of purified AR were incubated with 1nM of the indicated fluorescein-labeled probe in the binding buffer. The apparent $K_D$ for AR is ~25 nM under these conditions. Typical anisotropy values for free probe range from 40-45 mA and the probe bound to AR is ~100 mA.The probes used were fl-cARE, 5'fl-CTA GAT TAC GGT ACA TGA TGT TCT TAC TCA-3', fl-c3ARE, 5'fl-CTA GAT TAC AGT ACG TGA TGT TCT TAC TCA-3', and fl-PRE/GRE, 5'-fl- CTA GAT TAC AGA ACA ATC TGT TCT TAC TCA.

**Figure 3.3**

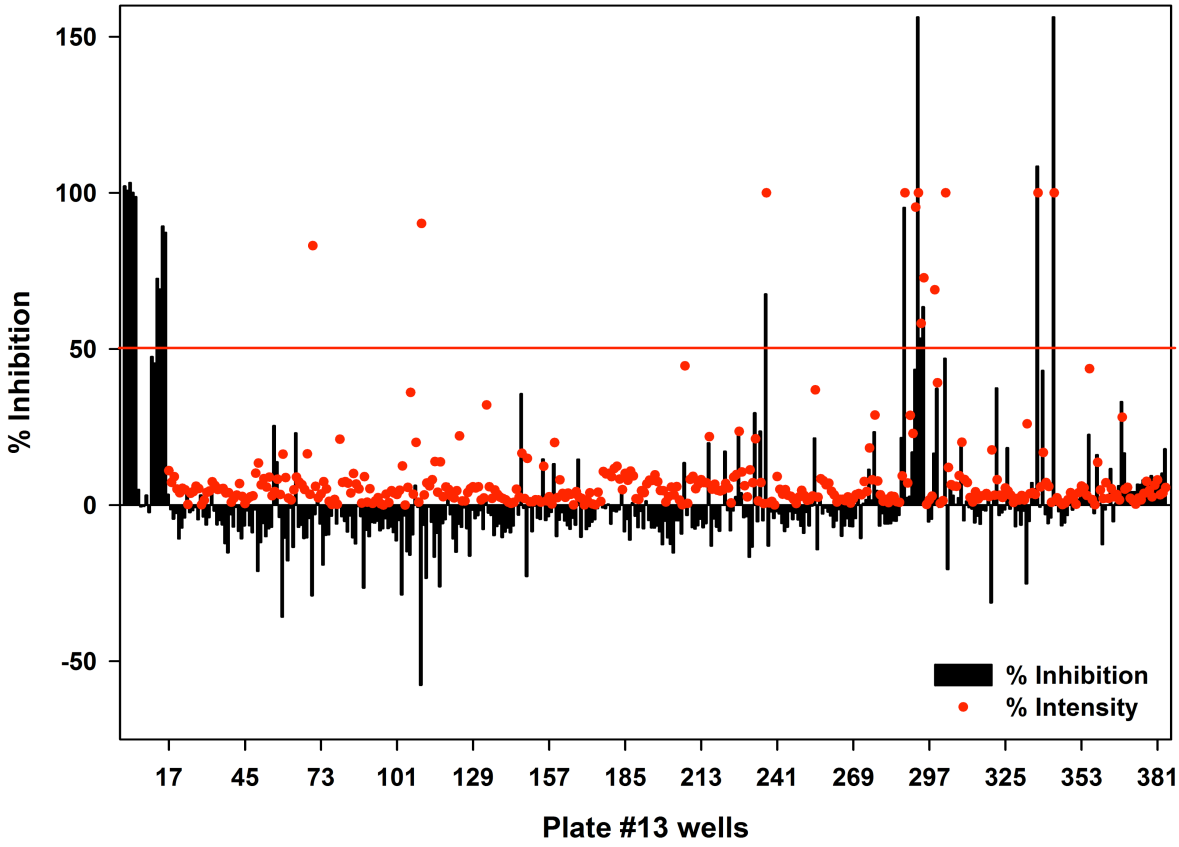



FIGURE 3.3. **A sample plate of FAMA based HTS for AR.**
45 nM AR was bound to 1 nM flcARE to obtain ~80% of maximum binding. 100 nl each of the small molecules from the library were added using a pin transfer apparatus to a final concentration of 2.5 µM and incubated for 15 minutes at room temperature. The effect of each compound, measured in % inhibition or % activation was calculated with respect to the DMSO control wells with AR:flcARE that were set at 0%. The black bars represents % inhibition calculated for individual wells. The first 16 wells contain various controls. Wells 1-5 contain 1 nM flcARE probe alone.Wells 6-10 have 45 nM AR with 1 nM flcARE. The next six wells are positive control wells spiked with 10 mM, 5 mM and 2.5 mM BPS, a zinc-chelator in duplicate. Intrinsic fluorescence intensity of some of the small molecules (red circles, % intensity), which is measured as part of each assay, is responsible for several of the most dramatic changes seen in the screening data.

**Figure 3.4**

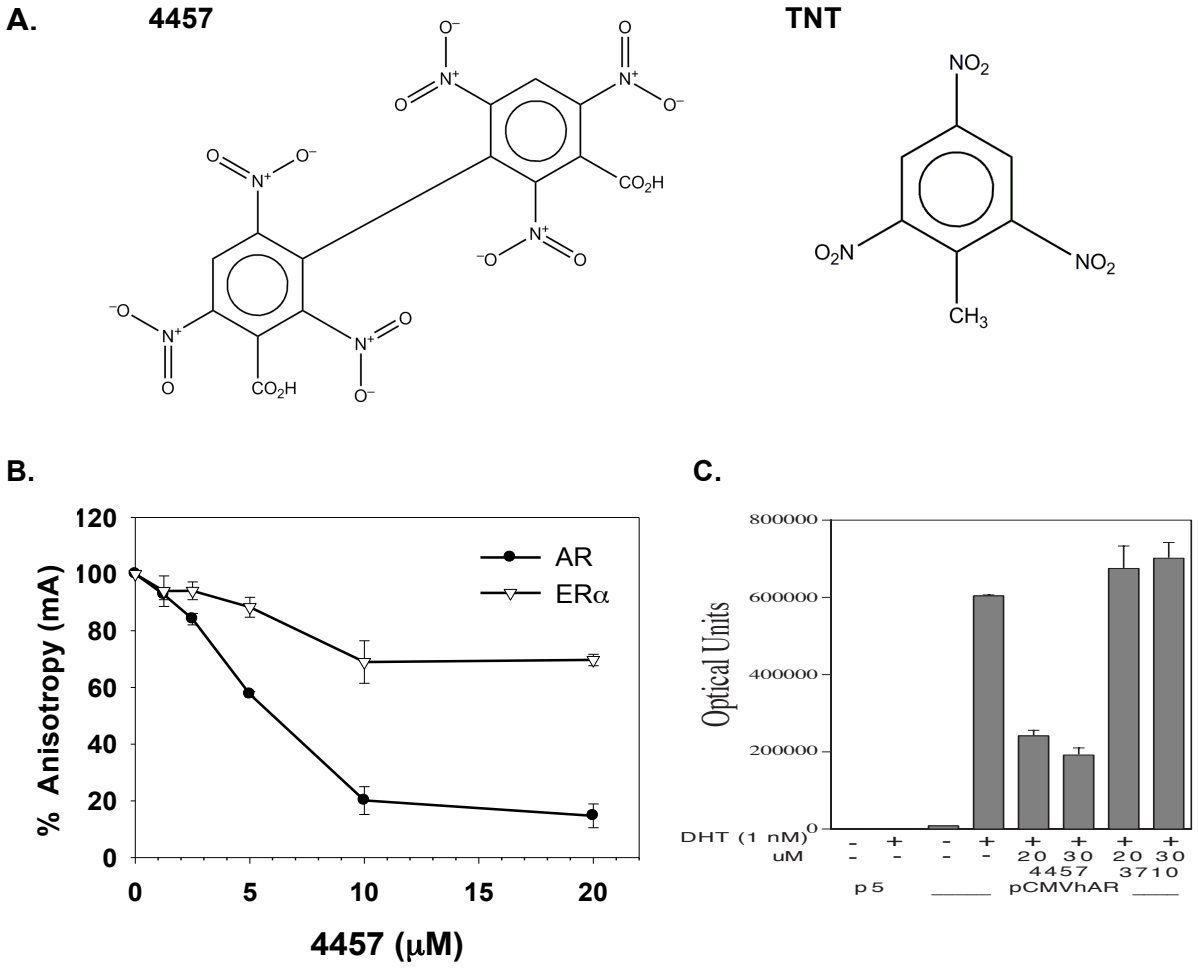



FIGURE 3.4. **Structure and dose response curve for 4457, in FAMA and cells.**
A. Chemical structure of 4457 compared to trinitrotoluene (TNT). B. Fluorescence anisotropy assays were carried out at concentrations of AR and ERα that exhibit ~80% of maximal binding (~2X $K_M$; 40 nM AR and 5 nM ERα). The indicated concentrations of 4457 were incubated with AR (filled circles), or ERα (open triangles) in reactions containing 1 nM flcARE or 1 nM flcERE for 15 min. and anisotropy was measured. Anisotropy in the DMSO control was set equal to 100%. The anisotropy change without inhibitor is ~60 mA for assays containing AR and ~35 mA for assays containing ER. The data represents the average of 4 independent experiments ± SEM. C. Effects of 20 and 30 μM 4457 on transactivation by AR in cells. HeLa cells were transfected with 10 ng pCMVhAR and 0.25 μg PSA-Enh-Luc using FuGENE-6 (Roche). Cells were incubated in serum-free medium in the presence or absence of 1 nM DHT and 4457 for 24 h and luciferase activity determined using a BMG Labtech Lumistar Galaxy automated luminometer.

**Figure 3.5**

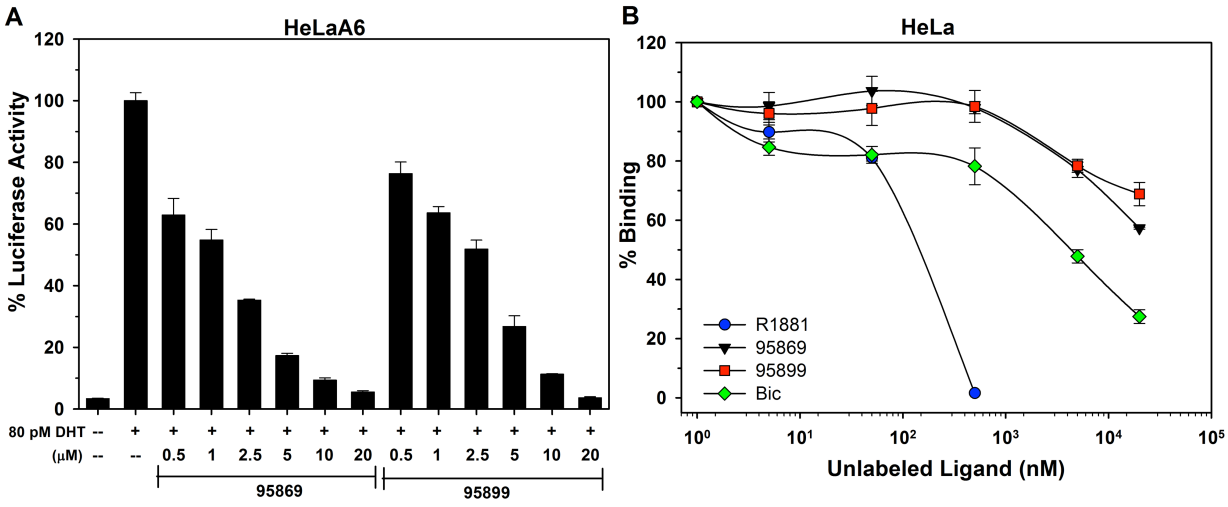
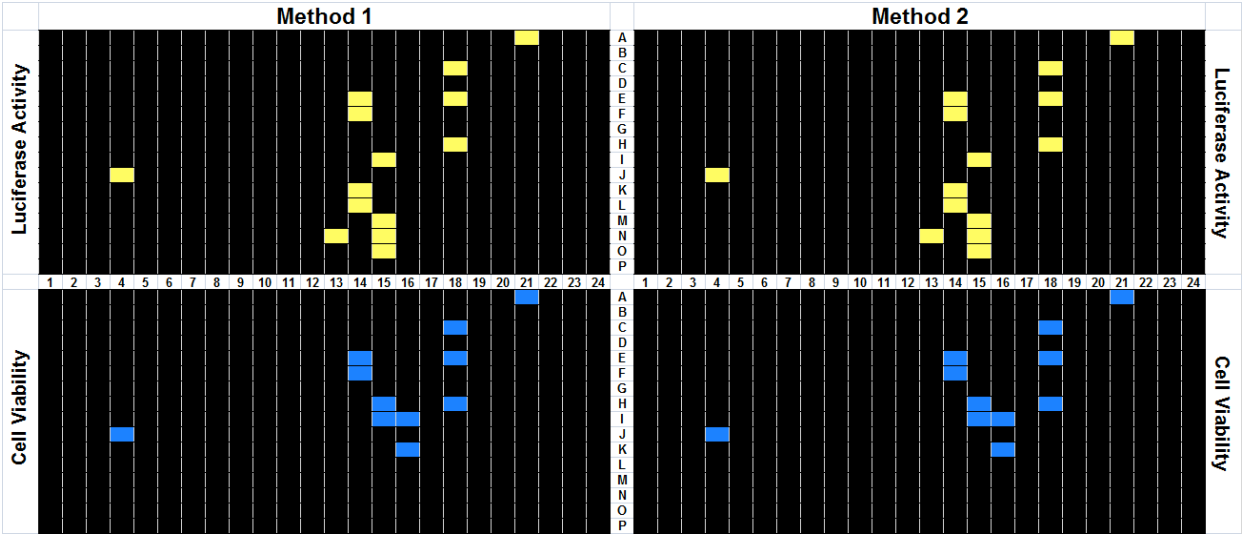



FIGURE 3.5. **Theophylline derivatives 95869 and 95899, reduces AR transactivation by competing with androgen for binding AR.** A. HeLaA6 cells were seeded in 24-well plates and maintained for 24 h in medium containing 80 pM DHT and the indicated concentrations of compounds 95869 or 95899. Data represent the average of triplicate experiments ± SEM. B. Competitive radioligand binding assay in COS cells. Relative binding affinity of various ligands for AR was determined using 5 nM [$^3$H]R1881 and a range of ligand concentrations (1-20,000 nM). Data are the average of duplicate experiments ± mean error.

**Figure 3.6**

**A**




**B**

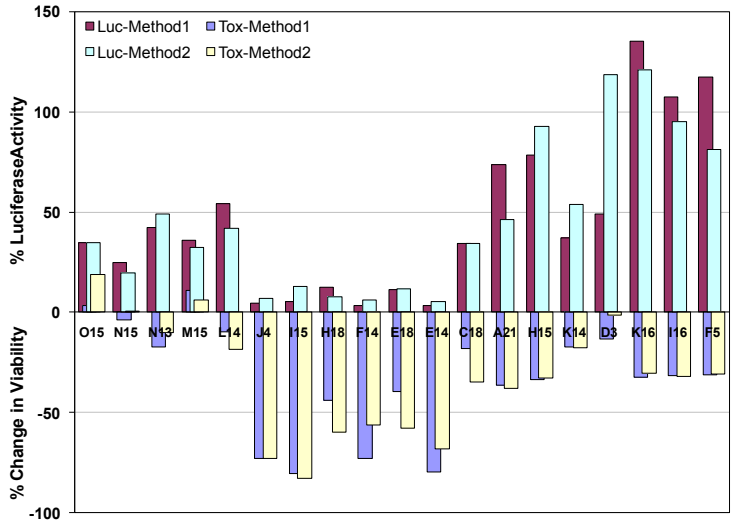



FIGURE 3.6. **A Hit map comparing both luciferase activity and cell viability of compounds from a single HTS library plate assayed using Methods 1 and 2.** Compounds on this representative 384-well plate that exerted >50% inhibition of luciferase activity are highlighted in yellow, while those that caused >20% reduction in cell viability are shown in blue. Wells treated with 5 nM DHT + DMSO (vehicle) were set to 100% in both assays. Luciferase inhibition and toxicity exerted by the small molecules were similar when the compounds were added after the cells had had one day to attach and recover (Method 1), or when the compounds were added along with the cells (Method 2). We chose Method 2 for large-scale screening.

**Figure 3.7**

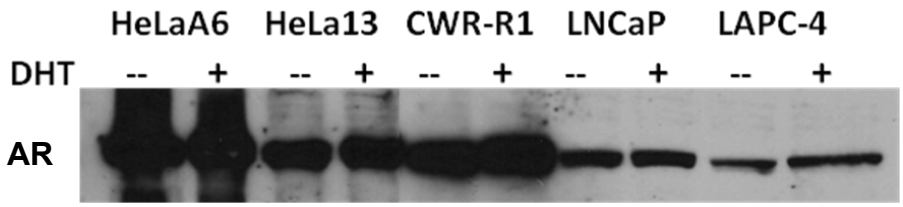



FIGURE 3.7. **Comparison of AR level and activity between HeLaA6 and HeLa13 cell lines.** Various cells were plated in medium containing 5% CD-FBS and stripped for at least 3 days. Treatment medium containing ethanol (vehicle) or 10 nM DHT was then added to the cells, incubated for 24 hours and protein extracted. Equal amounts of protein were loaded on 10% polyacrylamide gels, analyzed by western blotting using AR antibody.
Data in figure 3.7: Elizabeth M Wilson

**Figure 3.8**

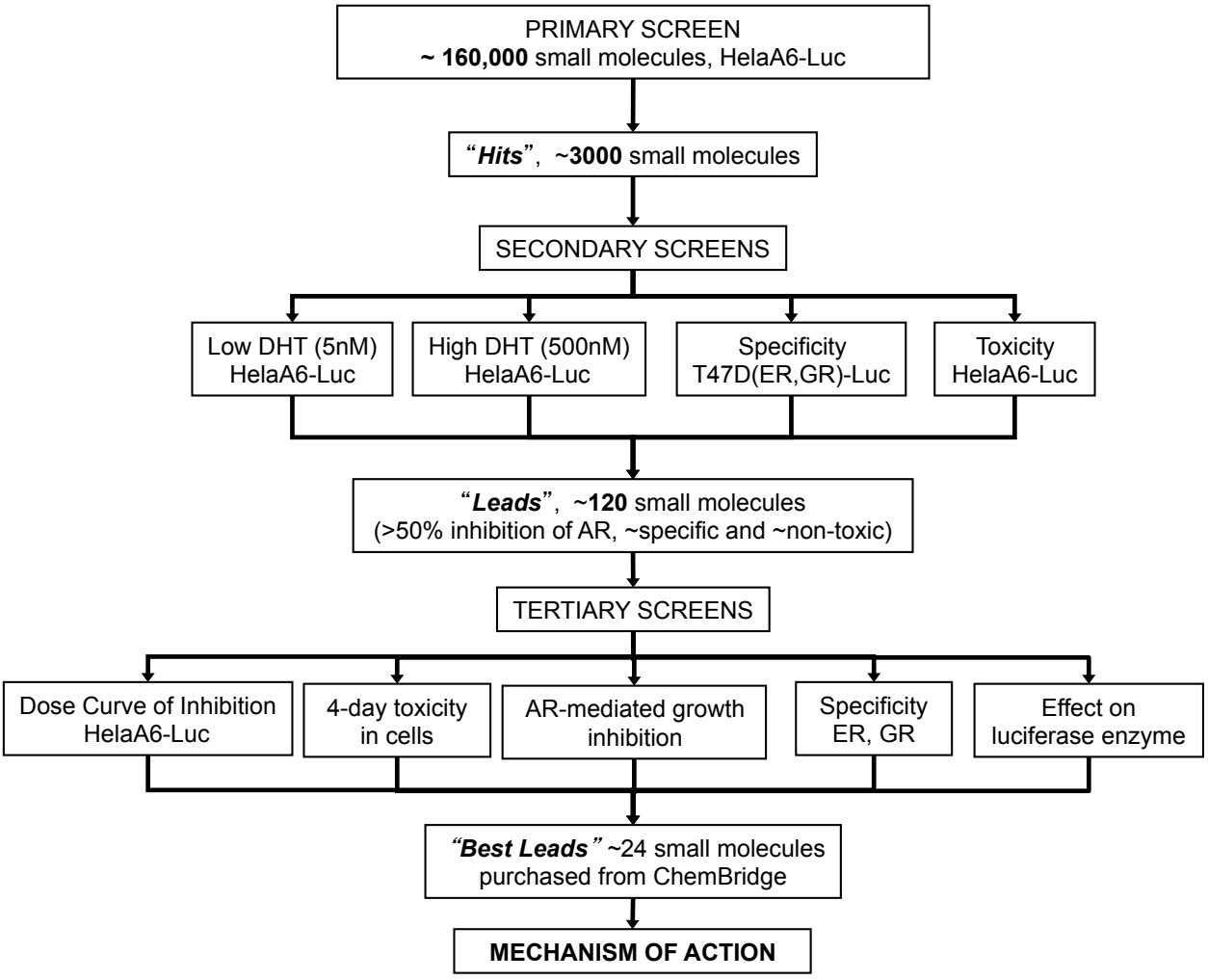



FIGURE 3.8. **Schematic diagram of the various steps involved in cell-based HTS for identification and characterization of small molecule inhibitors of AR action.**

# CHAPTER 4
# A SMALL MOLECULE INHIBITOR THAT REDUCES RECRUITMENT OF ANDROGEN RECEPTOR TO ANDROGEN RESPONSIVE GENES[2§]

The androgen receptor (AR) has a critical role in the growth and progression of androgen-dependent and castration-resistant prostate cancers. To identify novel inhibitors of AR transactivation that block growth of prostate cancer cells, a luciferase-based high throughput screen of ~160,000 small molecules was performed in cells stably expressing AR and a prostate-specific antigen (PSA)-luciferase reporter. CPIC (1-(3-(2-chlorophenoxy) propyl)-1H-indole-3-carbonitrile) was identified as a small molecule that blocks AR transactivation to a greater extent than other steroid receptors. CPIC inhibited AR-mediated proliferation of androgen-sensitive prostate cancer cell lines, with minimal toxicity in AR-negative cell lines. CPIC treatment also reduced the anchorage-independent growth of LAPC-4 prostate cancer cells. CPIC functioned as a pure antagonist by inhibiting the expression of AR-regulated genes in LAPC-4 cells that express wild-type AR, and exhibited weak agonist activity in LNCaP cells that express the mutant AR-T877A. CPIC treatment did not reduce AR levels or alter its nuclear localization. We used chromatin immunoprecipitation to identify the site of action of CPIC. CPIC inhibited recruitment of androgen-bound AR to the *PSA* promoter and enhancer sites to a greater extent than bicalutamide. CPIC is a new therapeutic inhibitor that targets AR-mediated gene activation with potential to arrest the growth of prostate cancer.

## INTRODUCTION

Androgens, such as testosterone and dihydrotestosterone (DHT), mediate their biological effects through the nuclear androgen receptor (AR). Binding of high-affinity androgens to the ligand-binding domain of AR induces the interdomain amino and carboxy-terminal (N/C) interaction (1-3). Activated AR translocates and accumulates in the nucleus, where it binds to specific androgen response elements (AREs) in the promoter and enhancer regions of androgen-

[2] This research was originally published in Journal of Biological Chemistry. MT Cherian, EM Wilson and DJ Shapiro, "A competitive inhibitor that reduces recruitment of androgen receptor to androgen responsive genes", *J. Biol. Chem.*, 2012 © the American Society for Biochemistry and Molecular Biology.

§ Disclosure: The University of Illinois at Urbana-Champaign has filed a novel use patent that claims CPIC.

regulated genes, and initiates transcription by recruiting multiple coregulators and the basal transcription machinery in a sequential and cyclic fashion (4, 5). Inhibitors of AR-mediated transcription include selective AR modulators (SARMs) such as the prostate cancer therapeutic, bicalutamide (Casodex) and the experimental drug MDV3100. Bicalutamide (Bic) and MDV3100 inhibit AR transcriptional activity by competing with androgens for binding to the ligand-binding domain of AR (6, 7). Bicalutamide can exhibit some agonist activity in cells containing mutant AR or expressing high AR levels (8, 9).

Androgens play a central role in the growth and development of the normal prostate gland and in the proliferation and progression of prostate cancers (10, 11). Patients with low-grade tumors benefit from primary therapies for prostate cancer, including radical prostatectomy and androgen deprivation or anti-androgen therapy. However, these treatment strategies are much less effective for long-term treatment of high-grade tumors (Gleason score ≥7) with elevated recurrence rates after primary therapy. In castration-resistant (or castration-recurrent) prostate cancer (CRPC), there may be higher levels of AR (12), increased expression of AR-regulated genes (13-15), and AR coregulators such as MAGE-A11 and SRC/p160 coactivators (16-19), suggesting that these cancers remain dependent on AR. Several types of evidence support the continuing role of AR in CRPC. Many of the genes induced by androgens in androgen-dependent prostate cancer xenografts become elevated in CRPC (20). Recent studies show that many advanced prostate cancers fuel their growth by synthesizing their own androgens (21). AR continues to be a focus for new drug development and it remains important to identify antagonists that block AR transcriptional activity (22). The importance of new approaches to targeting androgens and AR in CRPC is illustrated by Abiraterone acetate/Zytiga, an androgen synthesis inhibitor that prolongs survival in patients with advanced prostate cancer (23).

To search for new AR antagonists, we developed and implemented a cell-based high-throughput screen of ~160,000 small molecules using HeLa cells that stably express AR and an androgen-inducible PSA-luciferase reporter. The relatively high levels of AR in these cells and the high concentration of androgen we used in the screens, rendered bicalutamide largely ineffective as an AR antagonist. Here we describe 1-(3-(2-chlorophenoxy)propyl)-1H-indole-3-carbonitrile (CPIC), a lead compound that emerged from our screen. CPIC is a potent and selective AR antagonist that inhibits expression of endogenous AR-regulated genes and the androgen-dependent growth of prostate cancer cells. Chromatin immunoprecipitation (ChIP)

assays using the synthetic androgen methyltrienolone (R1881), showed that CPIC decreased AR occupancy at AREs of two prominent AR-regulated genes *PSA* and *TMPRSS2*, without affecting AR protein levels or AR nuclear translocation. In LNCaP cells that express a mutant AR-T877A and in LAPC-4 cells with wild-type AR, CPIC inhibited binding of R1881-AR to the PSA promoter and enhancer. The ability of CPIC to reduce recruitment of AR to multiple regulatory regions of androgen-responsive genes to a greater extent than bicalutamide suggests a new mode of action.

## EXPERIMENTAL PROCEDURES

***Chemical Libraries-*** The libraries screened were the ChemBridge MicroFormat small molecule library obtained from ChemBridge™ containing ~150,000 small molecules, the Marvel library developed at the University of Illinois by K. Putt and Hergenrother containing ~9,700 small molecules (24) and the NCI Diversity Set from NIH with ~1,990 small molecules.

***Plasmids-*** Expression vectors used have been previously described (25). pCMV-AR-(507–919) codes for the human AR DNA and ligand-binding domains, pCMV-AR-(1–503) codes for the AR $NH_2$-terminal domain. PSA-Enh-Luc containing the PSA upstream enhancer region was generously provided by Michael Carey (University of California, Los Angeles).

***Cell Culture-*** AR-positive cell lines included LNCaP and LAPC-4 human prostate cancer cells maintained in phenol-red free RPMI 1640 with 10% fetal bovine serum (FBS, Atlanta Biological, GA). LAPC-4 growth medium was routinely supplemented with 1 nM of the synthetic androgen R1881. Cells were transferred to RPMI 1640 containing 5% charcoal-dextran stripped FBS (CD-FBS) at least three days prior to plating for an experiment. CWR-R1 cells were grown in modified iMEM (GIBCO#10488-001) containing 2.5 g/l of glucose, 1.2 g/l niacinamide, 0.5 ml of insulin-transferrin-selenium (ITS, Roche #11074547001), 10 ng/mL epidermal growth factor (EGF) and 2% FBS. The cells were transferred to medium containing 2% CD-FBS without EGF 3-4 days before the experiment.

HeLa-AR1C-PSA-ARE-Luc-A6 (HeLaA6) cells selected with hygromycin and geneticin (G418) stably express human AR and a PSA-luciferase reporter gene containing the 5.8 kb PSA upstream enhancer and promoter region linked to the luciferase gene (26). HeLa-AR3A-PSA-ARE4-Luc-13 (HeLa13) cells selected with hygromycin and G418 stably express human AR and

a PSA-luciferase reporter gene containing a 4X multimerized PSA upstream enhancer ARE1 linked to the E4 TATA box and luciferase gene. Both HeLa-AR cell lines were maintained in MEM supplemented with 10% FBS. HeLaA6 cells were maintained under selection with 0.1 mg/ml hygromycin B and 0.5 mg/ml geneticin. HeLa13 cells were maintained under selection with 0.05 mg/ml hygromycin B and 0.5 mg/ml G418. Cells were transferred to medium containing 5% CD-FBS 3-4 days before the experiment. AR-negative cell lines included PC-3 human prostate cancer cells maintained in RPMI 1640 with 10% FBS, and DU145 human prostate cancer cells and MDA-MB-231 human breast cancer cells grown in MEM with 10% FBS.

Other cell lines included estrogen receptor α (ERα)-containing T47D-KBluc breast cancer cells expressing an $(ERE)_3$-luciferase reporter gene (27), maintained in RPMI 1640 containing 10% FBS. Three days before induction with 17β-estradiol ($E_2$), cells were transferred to medium containing 5% CD-FBS. T47D/(A1-2) cells stably express the human glucocorticoid receptor (GR) and contain a mouse mammary tumor virus (MMTV)-luciferase reporter (28) and were maintained in MEM, 5% FBS and 0.2 mg/ml G418. MMTV-Luc reporter is inducible by AR, PR and GR depending on the activating ligand used. Before the experiment, cells were transferred to the above medium containing 5% CD-FBS.

The RPMI base medium was supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM Hepes, pH 7.5, and 1 mM sodium pyruvate. MEM was supplemented with 10 mM HEPES, pH 7.4 and 2 mM L-glutamine. All cells were maintained at 37˚C in 5% $CO_2$ in growth medium containing 1% penicillin and streptomycin and 2-10% fetal bovine serum (FBS) (Atlanta Biological, Atlanta, GA) without phenol-red.

***Soft-agar colony formation assay-*** To assay anchorage-independent cell growth in soft agar, 1% and 0.7% Select Agar (Invitrogen) were prepared in water and warmed at 40°C before use. 1.5 ml of 0.5% bottom agar diluted in 2X RPMI 1640 medium was added to each well of a 6-well cell culture plate and allowed to solidify at room temperature. Top agar was prepared by dilution in warm medium. LAPC-4 cells were resuspended in 1.5 ml of 0.35% top agar at 5000 cells/well and plated in 3 wells for each condition. The plates were kept at room temperature for 30 min until the top agar solidified then 0.5 ml of medium containing the respective treatments was added on top of the agar. Culture medium containing the various treatments was changed every 3-4 days. Colonies were visible after 2 weeks in the hormone treated wells and counted at

day 28 using a dissecting microscope. Photographs of colonies were taken using a Zeiss AxioImager2 imaging system at 5X magnification.

***Reporter Gene Assays-*** At least 3 days before each experiment, cells were transferred to medium containing CD-FBS as described above. HeLaA6 and HeLa13 cells (50,000 cells/well) and T47DA/1-2 or T47D-KBluc cells (200,000 cells/well) were plated in 1 ml of medium in 24-well plates. After 24 h the indicated concentrations of $E_2$, DHT or dexamethasone (Dex) were added along with each inhibitor or DMSO (vehicle). After 24 h of treatment, cells were lysed in 100 µl of Passive Lysis Buffer (Promega, Madison WI) and luciferase activity was determined using BrightGlo firefly luciferase reagent (Promega). Unless otherwise mentioned, total DMSO (vehicle) concentration in all assays was maintained at or below 0.1%.

***Cell Growth and Viability Assays-*** To assay cell growth, 2,000 cells/well were plated in 96 well plates. LNCaP and LAPC-4 cells were maintained in CD-treated serum for at least 2-4 days prior to each experiment. All AR negative cell lines, MDA-MB-231, DU145 and PC-3, were plated in growth medium 24 h prior to treatment. Treatment medium containing vehicle or the indicated concentrations of R1881, with or without inhibitor compounds was added to the cells and incubated for the indicated number of days. Cell viability was determined using Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS).

***Transient Transfection-*** LAPC-4 cells were plated (200,000 cells/well in 1 ml) in 24-well plates in 5% CD-FBS 3 days prior to transfection. On the day of transfection, the medium was changed to 0.2 ml OPTI-MEM. DNA and Lipofectamine2000 (Invitrogen) were diluted in OPTI-MEM and incubated together for 20 min before adding to the well. A total of 500 ng DNA (400 ng PSA-Luc and 100 ng Renilla-Luc) was transfected into cells in each well at a DNA:Lipofectamine2000 ratio of 1:3. 24 h after transfection, 1 nM R1881 and the indicated concentrations of each inhibitor in DMSO, were added to the cells and incubated for 48 h. Cells were lysed in 100 µl of Passive Lysis Buffer (Promega, Madison WI) and luciferase activity was determined using Dual-Luciferase Reporter Assay (Promega #E1910). Transfections in HeLa cells were performed as described (29).

***Radioligand Binding Assay-*** Competitive radioligand binding assays were performed by expressing pCMV-AR in monkey kidney COS cells and incubating cell cultures for 2 h at 37˚C with [$^3$H]R1881 (17α-methyl-[$^3$H] methyltrienolone, 82 Ci/mmol) in the absence and presence of competitor ligands (30, 31).

***Endogenous Gene Expression-*** LNCaP and LAPC-4 cells were seeded into 6-well plates and grown for 3-4 days in medium containing 5% CD-FBS. Cells were then treated with ethanol or R1881, along with the indicated concentrations of the inhibitor for 24 h. RNA was extracted and purified using the Qiagen RNeasy kit. cDNA was prepared from 1 μg of RNA with M-MuLV reverse transcriptase from New England BioLabs. Diluted cDNA was used to perform quantitative RT-PCR using SybrGreen (ABI Thermocycler) with actin as the internal standard. Primers for qRT-PCR were: β-actin forward primer 5'- TGT CAC CAA CTG GGA CGA CA and reverse primer 5'- GGG GTG TTG AAG GTC TCA AA; PSA (kallikrein 3) forward primer 5'- GGT GAC CAA GTT CAT GCT GTG and reverse primer 5'- GTG TCC TTG ATC CAC TTC CG; TMPRSS2 forward primer 5'- TAG TGA AAC CAG TGT GTC TGC and reverse primer 5'- AGC GTT CAG CAC TTC TGA GGT CTT (6).

***Western Blot-*** Cells were plated at 300,000 cells/well in 6-well plates in medium containing 5% CD-FBS. The medium was changed on day 2, and on day 4 the cells received fresh medium containing the indicated treatments. Whole cell extracts were prepared after 24 h of treatment using 1X RIPA buffer (Millipore, CA) containing complete mini protease inhibitor cocktail (Roche, Germany). 30 μg of protein per lane was analyzed on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (GE Healthcare). AR protein was detected using AR antibody AR (441) (sc-7305, Santa Cruz, CA), internal control α-tubulin was detected using monoclonal antibody T1699 (Sigma) and β-actin was detected using antibody A1978 (Sigma).

***Chromatin Immunoprecipitation (ChIP) -*** LNCaP or LAPC-4 cells were grown in 5% CD-FBS for 4 days and pretreated with 10 μM CPIC, Bic or DMSO as control for 1 h before treatment with 1 nM R1881 or vehicle (ethanol) for 4 h. Proteins were cross-linked with 1% formaldehyde for 10 min. Cell extracts were digested for 10 min with 50 U of Micrococcal Nuclease (New England BioLabs) at 37ºC and further sonicated to yield sheared DNA fragments with an average length of 200-1000 base pairs. The sonicated samples were pelleted by centrifugation and the supernatant was diluted 5-fold with ChIP dilution buffer (0.01% SDS, 1.1 % Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl and protease inhibitor cocktail). 50 μl of diluted supernatant was reserved as input (10%) for each treatment. The samples were precleared with 50 μl of Protein A-Sepharose™ 4 Fast Flow (GE Healthcare) in ChIP dilution buffer (1:1), pre-blocked with 200 μg/ml sheared herring sperm DNA and 500

μg/ml BSA. The samples were then divided and the remaining proteins were incubated with either 2 μg of anti-AR (C19), 2 μg of anti-RNA polymerase II (clone CTD4H8, Millipore), or control mouse IgG overnight at 4˚C. The antibody-protein-DNA complex was precipitated by incubating with 50 μl Protein A-Sepharose™ beads for 2 h at 4˚C. The protein-DNA complex was eluted from the beads with elution buffer (1% SDS, 0.1 M $NaHCO_3$). Cross-links were reversed and DNA was eluted from the protein-DNA complexes by adding 200 mM NaCl and incubating overnight at 65˚C. Protein was digested using Proteinase K and incubating at 45˚C for 2 h. DNA was recovered and purified. Quantitative RT-PCR was performed to determine the change in AR and RNA polymerase II (RPol II) occupancy at various sites of AR binding. The double negative controls were nonspecific antibody (normal mouse IgG) and primers coding for intergenic regions that do not interact with AR. Thermal cycling conditions were 95ºC for 10 min, followed by 50 cycles of 25 s at 95ºC, 30 s at 60ºC and 30 s at 72ºC. Primers used were: PSA enhancer ARE forward primer 5'- ACC TGC TCA GCC TTT GTC TCT GAT and reverse primer 5'- AGA TCC AGG CTT GCT TAC TGT CCT; PSA promoter ARE forward primer 5'- CCT AGA TGA AGT CTC CAT GAG CTA CA and reverse primer 5'- GGG AGG GAG AGC TAG CAC TTG; Middle region forward primer 5'- CTG TGC TTG GAG TTT ACC TGA and reverse primer 5'- GCA GAG GTT GCA GTG AGC C (32-34).

***Statistical Analysis-*** Results are expressed as mean ± SEM of at least three independent experiments. Significance was established when $p<0.05$. Student's T-test was used for comparison of the means between two groups.

## RESULTS

***Establishment of Stable HeLa Cell Lines -*** For this work, HeLaA6 (26) and HeLa13 cells were established to stably express PSA-ARE-Luc reporter genes and AR at levels similar to or greater than LNCaP and LAPC- 4 cells. HeLaA6 cells express considerably more AR protein than prostate cancer cell lines like LNCaP or LAPC-4 (35) (Fig. 4.1A). The higher level of AR results in 10-40 μM bicalutamide acting as an agonist in HeLaA6 cells (Supplemental Fig. 4S.1A) and in this regard they resemble castration-resistant prostate cancer (CRPC). To more nearly mimic the environment of early-stage prostate cancer, we established the HeLa13 cell line that stably expresses AR protein at levels similar to LNCaP and LAPC-4 cells (Fig. 4.1A). There

is a 20-fold difference in the dose response curves between the two cell lines. The half-maximal effective DHT concentration ($EC_{50}$) was ~50 pM in the HeLaA6 cells compared to ~1 nM in the HeLa13 cells (Fig. 4.1B).

***Cell-based High-throughput Screening-*** Feasibility for the cell-based high-throughput screen was established using a combination of manual and robotic steps. Details of the screening procedure are elaborated in Chapter 3. To identify new small molecule inhibitors of AR action, we performed a luciferase-reporter based high-throughput screen using HeLaA6 cells and ~160,000 small molecules in several libraries at the University of Illinois High-Throughput Screening Facility.

***Identification of CPIC as a Lead Compound -*** To minimize the detection of moderate affinity competitor ligands, HeLaA6 cells were assayed in the presence of 5 nM DHT to fully saturate AR (see Fig. 4.1B). The lead inhibitor to emerge from our studies, CPIC (Fig. 4.2A), was subsequently shown to be a competitive inhibitor of androgen binding to AR. We evaluated the potency of CPIC in cells expressing high (HeLaA6) and moderate (HeLa13) levels of AR. The potency of CPIC in HeLaA6 and HeLa13 cells was compared to bicalutamide, a competitive AR antagonist that inhibits androgen binding to AR. CPIC elicited a concentration dependent inhibition of AR-induced luciferase activity in the HeLaA6 cells with an $IC_{50}$ of 5 μM, and was much more effective in HeLa13 cells with an $IC_{50}$ of 0.09 μM (Fig. 4.2B). In contrast to bicalutamide, which had substantial agonist activity in HeLaA6 cells in the absence of DHT, CPIC lacked agonist activity in these cells (Supplemental Fig. 4S.1) and thus functioned as a pure antagonist.

To evaluate the potency of CPIC in prostate cancer cells containing wild-type AR, LAPC-4 human prostate cancer cells were transfected with PSA-luciferase reporter. CPIC effectively inhibited luciferase activity induced by R1881-AR with an $IC_{50}$ of 1-5 μM (Fig. 4.2C).

***CPIC Competes with Androgen for Binding to AR and Disrupts the AR N/C Interaction-*** The ability of CPIC to compete with R1881 binding to AR was evaluated in cell-based assays. In competitive radiometric binding assays performed using 5 nM [$^3$H]R1881, CPIC competed for binding to AR (Fig. 4.3A). If CPIC is a competitive inhibitor of AR, increasing the hormone concentration should reduce the ability of CPIC to bind AR and block its action. To test this, we varied the DHT concentration by 100-fold and tested the ability of CPIC to inhibit PSA-Luc in HeLaA6 cells. Increasing the concentration of DHT from 0.1 nM to 10 nM increased the $IC_{50}$ for

inhibiting DHT-AR induced PSA-Luc transcription by ~20 fold (Fig. 4.3B). These data demonstrate that one mechanism for CPIC inhibition of AR-mediated transcription is by competing with androgen binding to AR.

To evaluate the effect of CPIC on the interdomain AR N/C interaction, we performed mammalian 2-hybrid assays using constructs containing the N- and C-terminal regions of AR. Transfections were performed in HeLa cells using pCMV-AR-(1-503) (the AR $NH_2$-terminal region), pCMV-AR-(507-919) ($NH_2$-terminal deletion of AR) and PSA-Enh-Luc reporter plasmids in the absence and presence of 10 nM DHT. Bicalutamide and other antagonists that bind to the ligand-binding pocket of AR disrupt this crucial interaction and alter receptor structure and function (36, 37). CPIC was substantially more effective than bicalutamide in inhibiting the androgen-induced AR N/C interdomain interaction (Fig. 4.3C).

***CPIC is a Relatively Specific Inhibitor of AR-Mediated Transactivation-*** The AR ligand-binding domain shares >50% sequence homology with the GR ligand-binding domain (38). We therefore evaluated the effect of CPIC on transcription by GR and ERα in cell lines stably expressing reporter genes. Inhibition of ERα was evaluated in T47D-KBluc cells that express endogenous ERα and are stably transfected to express an $(ERE)_3$-luciferase reporter. Inhibition of GR was evaluated in T47D/(A1-2) cells that stably express GR and a mouse mammary tumor virus (MMTV)-luciferase reporter gene. Fig. 4.4 shows that CPIC did not inhibit GR transcriptional activity and modestly inhibited ERα activity at 20 μM CPIC. The results suggest that CPIC is a relatively specific inhibitor of AR-mediated transcription.

***CPIC Inhibits AR-Dependent Proliferation of Prostate Cancer Cells-*** We evaluated the effect of CPIC on the proliferation of AR positive prostate cancer cell lines. LAPC-4 cells contain wild-type AR. LNCaP cells, the most widely used androgen-sensitive cell line, contain high levels of the mutant AR-T877A and CWR-R1 cells contain mutant AR-H874Y, both of which are highly inducible by androgens and other steroids (9, 39). CPIC inhibited androgen-mediated AR-dependent growth of all 3 cell lines, with an $IC_{50}$ of ~2 μM in LNCaP cells (Fig. 4.5A), ~0.3 μM in LAPC-4 cells (Fig. 4.5B) and ~5 μM in CWR-R1 cells (Fig. 4.5C).

The specificity of CPIC inhibition of AR-mediated cell growth was tested using AR-negative PC-3 and DU145 prostate cancer cells and MDA-MB-231 breast cancer cells. CPIC had little effect on the growth of PC-3 or MDA-MB-231 cells (Fig. 4.5D). However at high concentrations, CPIC slowed, but did not arrest the proliferation of DU145 cells. Thus,

functional concentrations of <5 μM CPIC were relatively specific for inhibition of AR-mediated cell growth. We also evaluated several small molecules structurally related to CPIC. These small molecules lacked the combined potency and specificity of CPIC. One member of this structural family, PIC19.7, exhibited an excellent toxicity profile (Supplemental Fig. 4S.2), but had substantially lower potency than CPIC and inhibited ER mediated transactivation at high concentrations.

***CPIC Inhibits Anchorage-Independent Growth of LAPC-4 cells-*** Anchorage-independent growth is a hallmark of cancer cells. Growth in soft agar is often used to evaluate anchorage-independence of human prostate cancer cells. We tested the ability of CPIC to inhibit colony-formation of wild-type AR containing LAPC-4 cells grown in soft agar. LAPC-4 cells supplemented with medium containing 1 nM R1881 formed large colonies (>0.5 mm) after 4 weeks (Fig 6, R1881). The growth of these cells was completely inhibited in the presence of 10 μM CPIC as well as 10 μM Bic (Fig. 4.6). When colonies from all the wells of each treatment condition were counted, the R1881 treated plate contained 77 colonies/well mostly >0.5 mm in diameter. In comparison, there were no colonies >0.5 mm in diameter in the R1881+ CPIC treated wells, and on average 2 small colonies/well less than 0.5 mm in diameter. A similar effect was seen in the R1881+ Bic treated wells. The data indicate that CPIC inhibits androgen-stimulation of anchorage-dependent (Fig. 4.5) and anchorage-independent (Fig. 4.6) growth of prostate cancer cells.

***CPIC Inhibits Expression of Endogenous AR-Regulated Genes-*** To evaluate the effect of CPIC on endogenous gene expression, the levels of mRNAs for several well characterized androgen-regulated genes were measured in LNCaP and LAPC-4 cells. The *PSA* and *TMPRSS2* genes are highly induced by androgens acting through AR. In LNCaP cells, 10 μM CPIC exhibited weak agonist activity and blocked AR-mediated transcription of PSA and TMPRSS2 mRNAs in a dose-dependent manner with $IC_{50}$s of ~0.5 μM and 0.3 μM, respectively (Fig. 4.7A). In contrast to LNCaP cells, in LAPC-4 cells that express wild-type AR, CPIC was a pure antagonist and was more effective than bicalutamide in inhibiting induction of PSA and other genes (Fig. 4.7B and Supplemental Fig. 4S.3B). TMPRSS2 mRNA was minimally induced by androgen in LAPC-4 cells, and thus the effect of CPIC could not be evaluated (data not shown). CPIC also inhibited androgen-regulated expression of other genes including kallikrein 2 (KLK2) and TMEPAI in LNCaP and LAPC-4 cells (Supplemental Fig. 4S.3).

The AR-T877A mutant in LNCaP cells is activated by adrenal androgens, estrogens, and progestins, as well as many antiandrogens (39) which may explain the weak agonist activity of CPIC. Alternatively, higher levels of AR in LNCaP cells compared to LAPC-4 cells (Fig. 4.1A) or cell type specificity might be responsible for the weak agonist activity of CPIC. To test the effect of the AR-T877A mutation, transient transfections were performed in HeLa cells. For both wild-type AR and AR-T877A, CPIC did not induce luciferase activity above the no hormone control (Supplemental Fig. 4S.4). Since CPIC did not exhibit weak agonist activity in HeLaA6 cells containing high levels of wild-type AR (Supplemental Fig. 4S.1B), or in HeLa cells transfected with AR-T877A (Supplemental Fig. 4S.4), it seems likely that cell context contributes to the weak agonist activity of CPIC in LNCaP cells.

***CPIC Does Not Decrease AR Levels or Reduce Nuclear Translocation-*** CPIC could inhibit AR-mediated gene expression through multiple mechanisms (40) that include (a) increased AR degradation, (b) reduced nuclear localization of liganded-AR, (c) inhibition of AR recruitment to response elements on DNA, and (d) altered coregulator recruitment. Western blot analysis showed that CPIC had little or no effect on intracellular levels of AR (Fig. 4.8A).

We used fluorescent polyclonal antibody to visualize intracellular AR in LNCaP and LAPC-4 cells. AR was predominantly nuclear in the presence of 10 nM R1881. In LNCaP cells, when 10 μM CPIC or Bic was present for either 4 or 24 h, AR was predominantly localized in the nucleus (Fig. 4.8B and Supplemental Fig. 4S.5A). Staining DNA with DAPI showed nuclear co-localization of CPIC-bound AR in LAPC-4 cells (Supplemental Fig. 4S.5B). These data suggest that CPIC promotes nuclear localization of AR and might influence AR association with DNA.

***CPIC Inhibits AR Binding to Androgen-Responsive Genes-*** ChIP was used to evaluate the effect of CPIC on AR recruitment to regulatory regions of androgen-responsive genes in LNCaP and LAPC-4 cells. *PSA* and *TMPRSS2* are two well-characterized androgen-regulated genes with defined AREs in their promoter and enhancer regions (41, 42). In LNCaP cells, R1881 increased AR recruitment to the PSA enhancer and promoter regions (Fig. 4.9A&B) and the TMPRSS2 enhancer (Supplemental Fig. 4S.6A). RNA polymerase II was also recruited to these AREs in the presence of androgen-bound AR. Consistent with its weak agonist activity in LNCaP cells, with 10 μM CPIC alone, there was a small increase in association of AR with the PSA enhancer and promoter (Fig. 4.9A&B). Although there is diversity in the reported effects of bicalutamide on AR binding to the PSA enhancer in LNCaP cells (6, 43, 44), most reports suggest that

bicalutamide interferes with androgen action at the PSA promoter by altering AR interaction with coregulators rather than inhibiting AR DNA binding (34, 45, 46). We found that bicalutamide inhibited recruitment of R1881-bound AR to the PSA enhancer to a greater extent than the PSA-promoter (Fig. 4.9A&B). CPIC strongly inhibited recruitment of R1881-AR to the PSA-promoter, PSA-enhancer and the TMPRSS2-enhancer (Fig. 4.9A&B and supplemental Fig. 4S.6A).

Since CPIC has no effect on AR levels or nuclear localization (Fig. 4.8), the decrease in AR occupancy is likely an effect of CPIC on AR binding to AREs and not a result of reduced levels of nuclear AR. Consistent with its ability to reduce AR binding to the promoter and enhancer regions of PSA and TMPRSS2, CPIC also reduced recruitment of RPol II (Fig. 4.9C&D and Supplemental Fig. 4S.6B). The results suggest that CPIC inhibits AR binding to AREs.

In LNCaP cells, 10 μM CPIC exhibits weak agonist activity at the PSA gene (Figs. 7A and 9). To evaluate the effect of CPIC in cells in which it acts as a pure antagonist, we performed ChIP in LAPC-4 cells. Because LAPC-4 cells contain lower levels of AR relative to LNCaP cells, there are few instances of ChIP performed using these cells. In LAPC-4 cells, 10 μM CPIC in the absence of R1881 did not increase AR occupancy at the PSA enhancer or promoter (Fig. 4.10A&B) or the KLK2-enhancer (Supplemental Fig. 4S.6C). Bicalutamide had no effect on R1881-AR recruitment at the PSA promoter, whereas CPIC strongly inhibited R1881-AR recruitment to the PSA promoter (Fig. 4.10B). CPIC was somewhat more effective than Bic in reducing AR recruitment to the PSA enhancer (Fig. 4.10A). Consistent with its inhibition of AR binding at the *PSA* regulatory regions, CPIC also prevents recruitment of RPol II (Fig. 4.10C&D and Supplemental Fig. 4S.6D).

To investigate the effect of CPIC on interaction of coactivators to AR, we used mammalian 2-hybrid assays. Since this assay uses GAL4 DNA binding domain, we investigated the effects of CPIC on coactivator recruitment independent of its effects on AR binding to AREs in responsive genes. Previous work showed that TIF2 (GRIP1/SRC2) interacts with the AR ligand binding domain through its LXXLL motifs (1). In mammalian two-hybrid assays in HeLa cells, CPIC moderately reduces the interaction between the AR ligand-binding domain and the TIF2 fragment required for interaction with AR (Supplemental Fig. 4S.7). There was essentially no effect on TIF2 interaction at 5 μM CPIC and 50% inhibition at 10 μM CPIC.

We evaluated the relationship between the weak agonist activity of CPIC in LNCaP cells that induced PSA and TMPRSS2 mRNAs, and the extent to which CPIC enhances AR occupancy at regulatory sites in *PSA* and *TMPRSS2*. CPIC alone induced TMPRSS2 mRNA to ~24% of maximum and AR binding to the TMPRSS2 enhancer to ~26% of maximum (Fig. 4.7 and Supplemental Fig. 4S.6). There was also a correlation between the ability of CPIC to inhibit R1881 induction of PSA and TMPRSS2 mRNAs and its ability to inhibit binding of AR to the regulatory regions of these genes. These results suggest that CPIC acts by decreasing AR binding to AREs in androgen-responsive genes.

**DISCUSSION**

The importance of AR in the development and growth of prostate cancer make AR an important therapeutic target. AR has properties unique among the steroid receptor family that influence androgen-dependent gene regulation. The AR interdomain interactions and a more limited role of coactivator LxxLL motifs (1, 3) highlight significant differences between AR and other steroid receptors. One way to probe the mechanism of AR action is by identification and characterization of novel small molecule inhibitors. The identification of a new coactivator binding surface on AR using low potency small molecule inhibitors, unrelated to CPIC, identified by screening (47) supports the utility of using small molecules as probes of AR actions. New small molecule inhibitors also have the potential to be therapeutically relevant.

Molecular mechanisms leading to the development of prostate cancer resistance to antagonists are not fully understood. In a limited number of cases, AR mutations such as AR-T877A can confer resistance to antiandrogens that function as weak agonists (39). To identify new small molecule AR inhibitors, we developed and implemented a cell-based high-throughput screen. Since LNCaP cells contain mutant AR-T877A, and LAPC-4 cells contain wild-type AR but have a doubling time of ~3 days, these cell lines were unsuitable for a luciferase-based screen. Although assays for androgen-stimulated proliferation of LNCaP and LAPC-4 cells work well in a 96-well format, they did not reach the requisite level of precision and reproducibility needed for a high-throughput screen in 384-well plates. For both the primary screen and preliminary analysis of hits, HeLa cells stably expressing wild-type AR and a PSA-luciferase reporter were used. The classical approach to cell-based screening involves adding the test compounds to cells that are previously plated. We found a simpler approach, in which the

inhibitor and cells are added at the same time produced equivalent results and could be readily automated. Consistent with its properties in LNCaP cells stably transfected to express elevated levels of wild-type AR (6), bicalutamide was an agonist in the HeLaA6 cells that express high levels of AR compared to minimal agonist activity in HeLa13 cells with moderate levels of AR similar to prostate cancer cells. In contrast to bicalutamide, which did not inhibit the androgen-induced AR N/C interaction at the sub-micromolar concentrations tested, CPIC potently disrupted the androgen-dependent interdomain AR N/C interaction.

Based on our assays CPIC was selected for detailed evaluation. An effective small molecule inhibitor of AR should exhibit high potency and specificity. Our studies show that CPIC inhibits AR transcriptional activity, with little or no effect on GR or ERα under the same conditions where other small molecules robustly inhibited ERα and GR (26, 48). CPIC effectively inhibited androgen-AR-dependent proliferation of LNCaP, LAPC-4 and CWR-R1 prostate cancer cells. At relevant concentrations, CPIC had little or no effect on the proliferation of several AR negative cell lines. Anchorage-independent colony-formation is a characteristic of many cancers and CPIC nearly abolished the AR-dependent proliferation of LAPC-4 prostate cancer cells in soft-agar. Our studies therefore suggest that CPIC is a potent and selective inhibitor of AR action in prostate cancer cells.

Radioligand binding assays and gene expression studies using stably transfected cell lines indicate that high levels of CPIC compete with androgen binding. By far the most straightforward explanation of this data is that CPIC is a competitive inhibitor of androgen binding to AR. However, our data do not exclude the possibility that CPIC binds to AR outside of the ligand-binding pocket and induces an inactive AR conformation opposed to androgen binding. When very high concentrations of androgens are present, AR is predominantly in the active conformation, which suggests a competitor phenotype.

Although radioligand binding and cell-based assays show that CPIC competes with androgen for binding to AR, the weak agonist activity of CPIC in LNCaP cells did not result from the AR-T877A mutation. Cell context and high AR-T877A are likely responsible for the weak agonist activity of CPIC.

Interestingly, CPIC has a different site of action than two experimental competitive inhibitors of AR, MDV3100 (6) and ARN-509 (49). In part because MDV3100 required a

relatively high 100 mg/kg/d dose to elicit a maximum *in vivo* response, ARN-509 was produced that elicited a maximum response at 30 mg/kg/d. A major site of action of both MDV3100 and ARN-509 is inhibition of AR nuclear localization (6, 49). In contrast, CPIC has no effect on nuclear localization, and acts at the level of AR binding to regulatory regions in responsive genes.

CPIC exhibited weak agonist activity in LNCaP cells and modestly induced PSA and TMPRSS2 mRNAs in the absence of androgen. However, 10 μM CPIC and 10 μM bicalutamide alone had similar minimal effects on the proliferation of LNCaP cells. Tamoxifen, which competes with estrogens for binding to ER, exhibits partial agonist activity in stimulating gene expression in MCF-7 human breast cancer cells (50, 51), but is widely used in breast cancer therapy. Since the weak agonist activity of CPIC in LNCaP cells does not stimulate of LNCaP cell proliferation, CPIC has therapeutic potential.

The inability of CPIC to influence AR levels or block agonist-induced AR nuclear localization together with the results from ChIP assays indicate that the inhibitory effects of CPIC occur at the gene level. R1881 induced a ~27 fold increase in AR occupancy at the PSA enhancer, ~11 fold increase in occupancy at the PSA promoter and a ~18 fold increase in occupancy at the TMPRSS2 regulatory region. The extent to which CPIC acts as a weak agonist in LNCaP cells and induced PSA and TMPRSS2 mRNAs correlated with the extent to which CPIC enhanced AR occupancy at regulatory sites in *PSA* and *TMPRSS2*. These data suggest that CPIC-bound AR at the *PSA* and *TMPRSS2* genes is transcriptionally competent.

Consistent with the mRNA data, CPIC does not exhibit any weak agonist activity in LAPC-4 cells. In these cells, CPIC significantly reduced androgen-induced AR recruitment, and consequently reduced RNA polymerase II recruitment to the PSA promoter and enhancer regions. In contrast, bicalutamide in the presence of androgen did not inhibit AR occupancy at the PSA promoter in LNCaP cells (5), and has been reported to recruit corepressors to the promoter region (34, 45). Our data indicate that CPIC functions as an AR inhibitor by decreasing the interaction of AR with regulatory regions of androgen-responsive genes.

AR and other steroid receptors exhibit a high level of conformational flexibility. Small molecules such as CPIC and bicalutamide may elicit different AR conformations. Our analysis suggests that although CPIC is similar to bicalutamide in its ability to inhibit androgen-induced transcription at the *PSA* gene locus, they may evoke different conformational changes when

bound to AR and have different mechanisms of action. CPIC was much more effective than bicalutamide in the inhibition of R1881-AR binding to the PSA promoter. The weak agonist activity of CPIC in LNCaP cells could be due to an AR conformation in which binding to AREs is reduced but not eliminated, an altered coactivator population that stabilizes weak binding of CPIC-AR to AREs, or the existence of multiple CPIC-AR conformations, one of which binds DNA and one of which is unable to bind DNA. With its unique mode of action, CPIC is a new potential inhibitor of prostate cancer growth.

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**FIGURES**

**Figure 4.1**

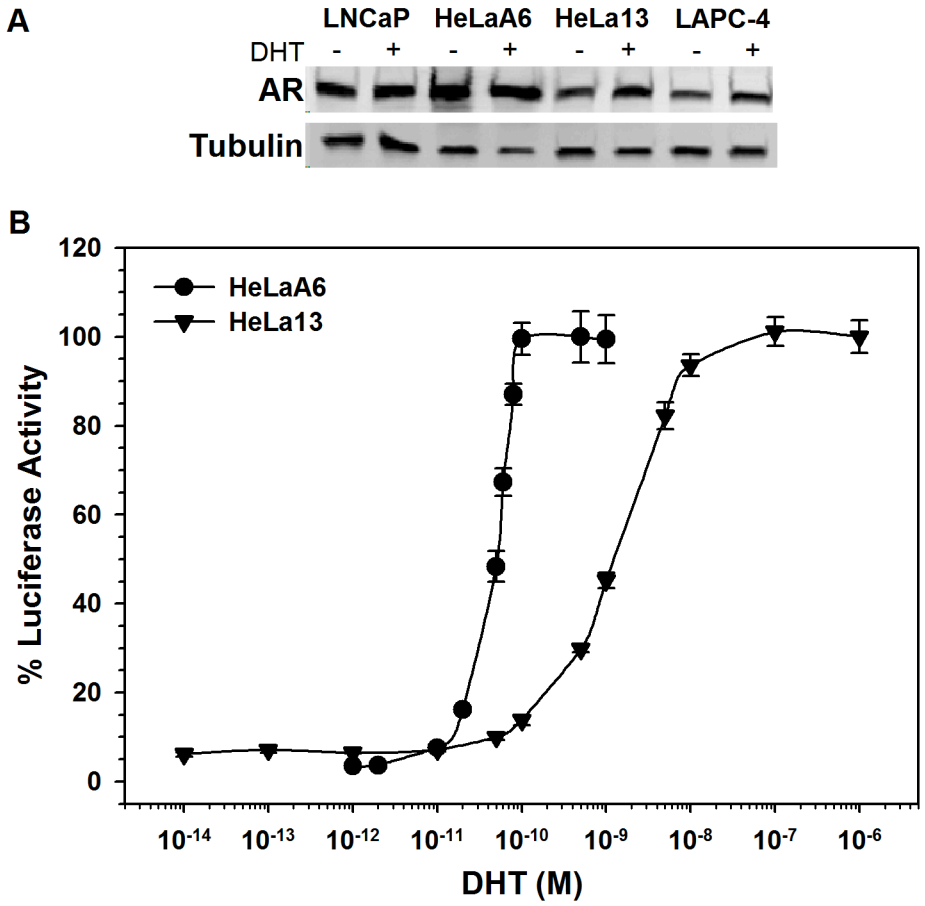



FIGURE 4.1. **Comparison of AR level and activity in HeLaA6 and HeLa13 cells.** *A*, Western blot showing AR levels in HeLaA6 and HeLa13 cells. After at least 3 days in medium containing 5% CD-FBS, ethanol (-) or 10 nM DHT (+) was added. After 24 h, the cells were harvested, protein was extracted and equal amounts of protein were fractionated on 10% polyacrylamide gels, analyzed by western blotting. Tubulin served as a loading control. *B*, Shown are the DHT dose-response curves for HeLaA6 and HeLa13 cells. HeLaA6 or HeLa13 cells were plated one day prior to treatment with the indicated concentrations of DHT or ethanol (vehicle) control. After 24 h of incubation, lysates were prepared and PSA luciferase activity measured in extracts from 3 wells of cells (Mean ± standard error of mean (SEM)). Activity in the presence of saturating DHT was set to 100%.

**Figure 4.2**

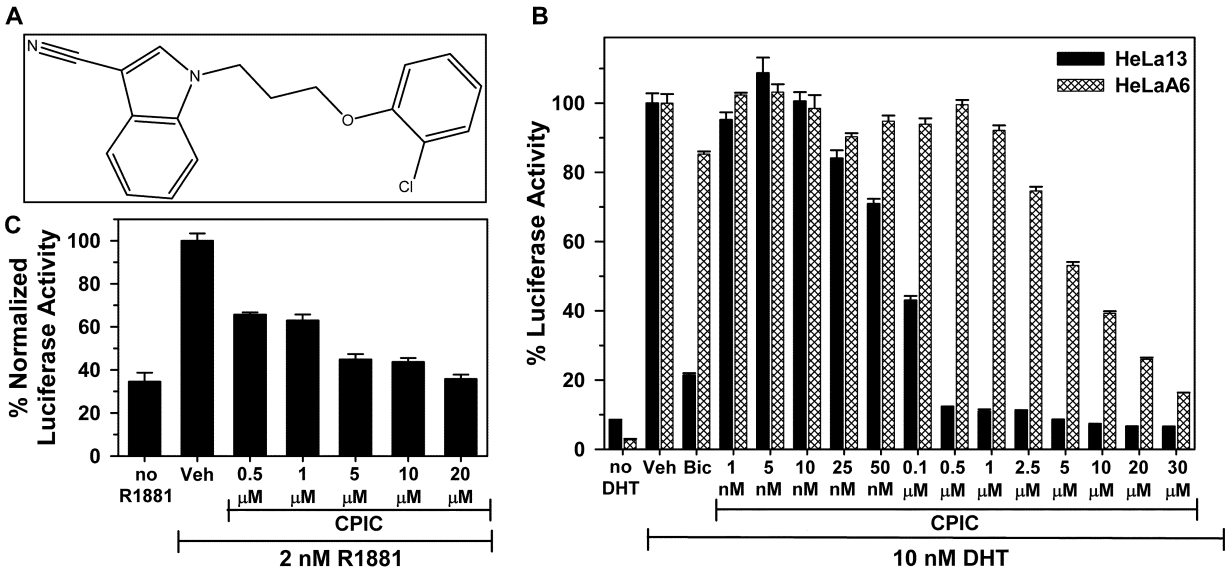



FIGURE 4.2. **CPIC inhibits androgen induction of PSA-luciferase activity.** *A*, Structure of CPIC. *B*, Dose-response studies of CPIC inhibition of DHT-AR induced PSA-luciferase in HeLaA6 and HeLa13 cells. Cells were seeded in 24-well plates and treated with medium +/- 10 nM DHT containing DMSO (Veh), the indicated concentrations of CPIC, or 10 μM bicalutamide (Bic) for 24 h and assayed for PSA luciferase activity. Activity of the reporter in the presence of 10 nM DHT and DMSO vehicle was set to 100%. Data represent the mean of 3 experiments ±SEM. *C*, LAPC-4 cells transiently transfected with 400 ng of PSA-Enh-luciferase plasmid and 100 ng CMV-Renilla luciferase were treated with the indicated concentrations of CPIC or DMSO in the presence of 2 nM R1881 for 48 h. Cell lysates were assayed for firefly-luciferase and normalized with Renilla-luciferase activity. Bars represent the mean of 3 experiments ±SEM.

**Figure 4.3**

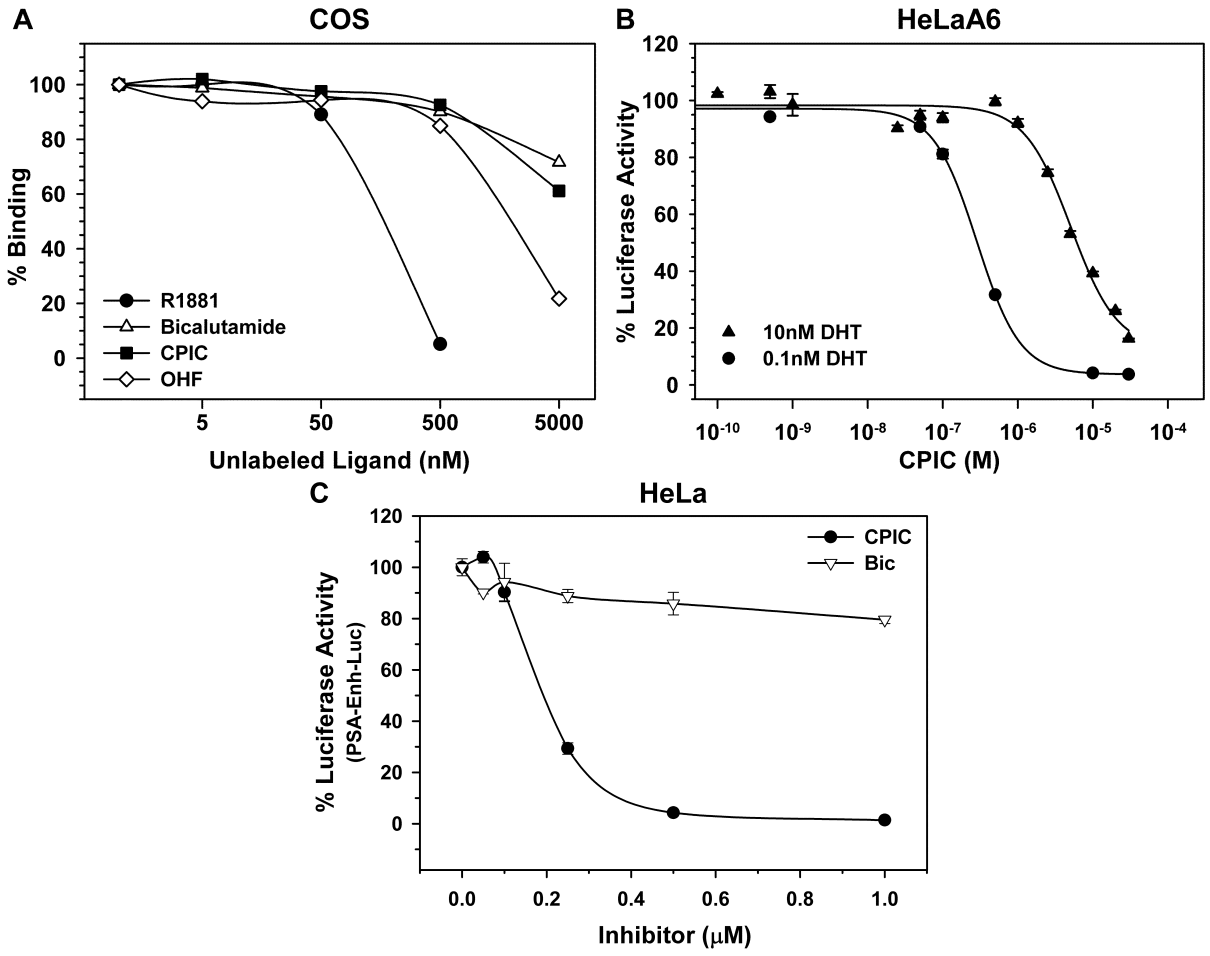



FIGURE 4.3. **CPIC competes with androgens for binding to AR and reduces the AR N/C interaction.** *A*, Competitive radioligand binding assay. Relative binding affinity of CPIC for AR was determined using 5 nM [$^3$H]R1881 and a range of CPIC concentrations as described (31). Data are the average of duplicate experiments. *B*, Effect of DHT concentration on CPIC inhibition of AR induced luciferase activity. HeLaA6 cells were seeded in 24-well plates and maintained for 24 h in medium containing 0.1 nM DHT (circle) or 10 nM DHT (triangle) and the indicated concentrations of CPIC. Data represent the average of triplicate experiments ± SEM. *C*, HeLa cells were transfected with 100 ng PSA-Enh-Luc reporter plasmid, 50 ng pCMV-AR-(1-503) and 50 ng pCMV-AR-(507-919). Cells were incubated in the absence or presence of 10 nM DHT and the indicated concentrations of CPIC or bicalutamide (Bic). Luciferase units of DHT-

treated wells were set at 100%. The luciferase activity is representative of two independent experiments.

Experimental Data in Figure 4.3C: EM Wilson

**Figure 4.4**

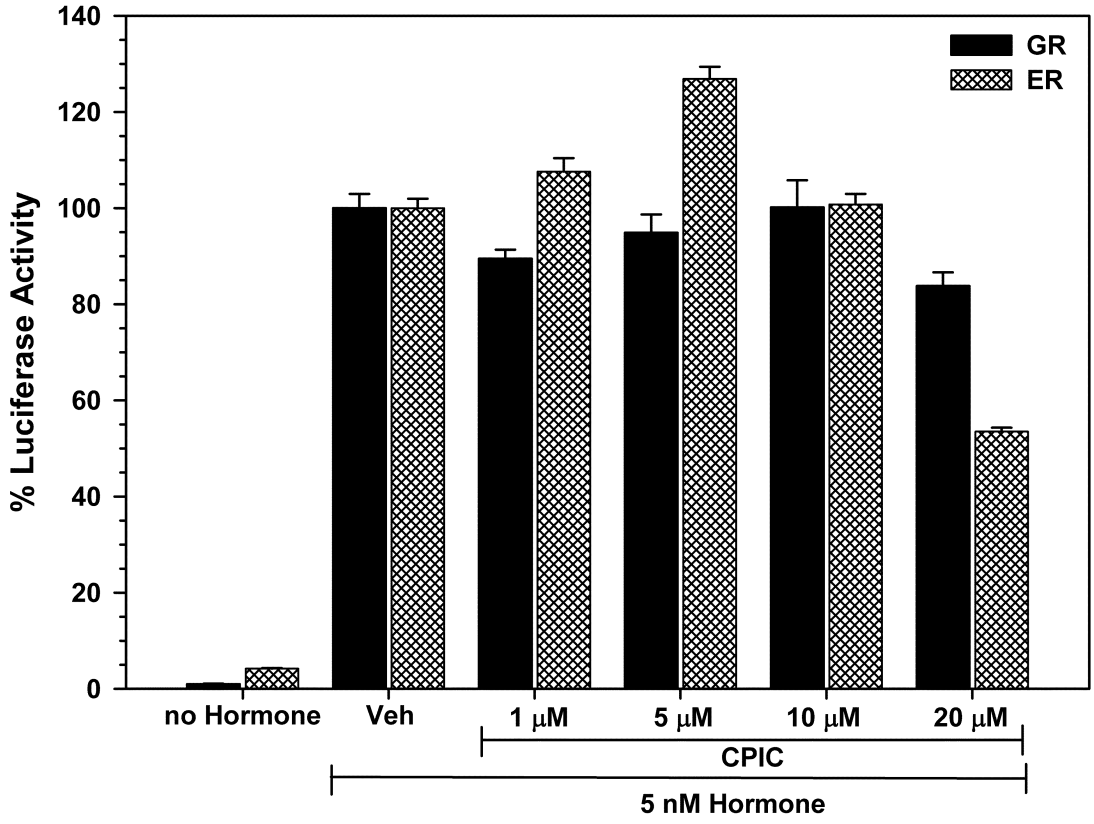



FIGURE 4.4. **Partial inhibition of ERα activity at high concentrations of CPIC.** T47D-KBluc and T47D/(A1-2) cells were seeded in 24-well plates in medium containing 5% CD-FBS. After the cells attached, treatment medium was added with 5 nM of 17β-estradiol for ER in T47D-KBluc cells, and 5 nM dexamethasone for GR in T47D/(A1-2) cells and the indicated concentrations of CPIC (or DMSO vehicle). After 24 h cell lysates were analyzed for luciferase activity. Data are the average of triplicate experiments ± S.E.M.

**Figure 4.5**

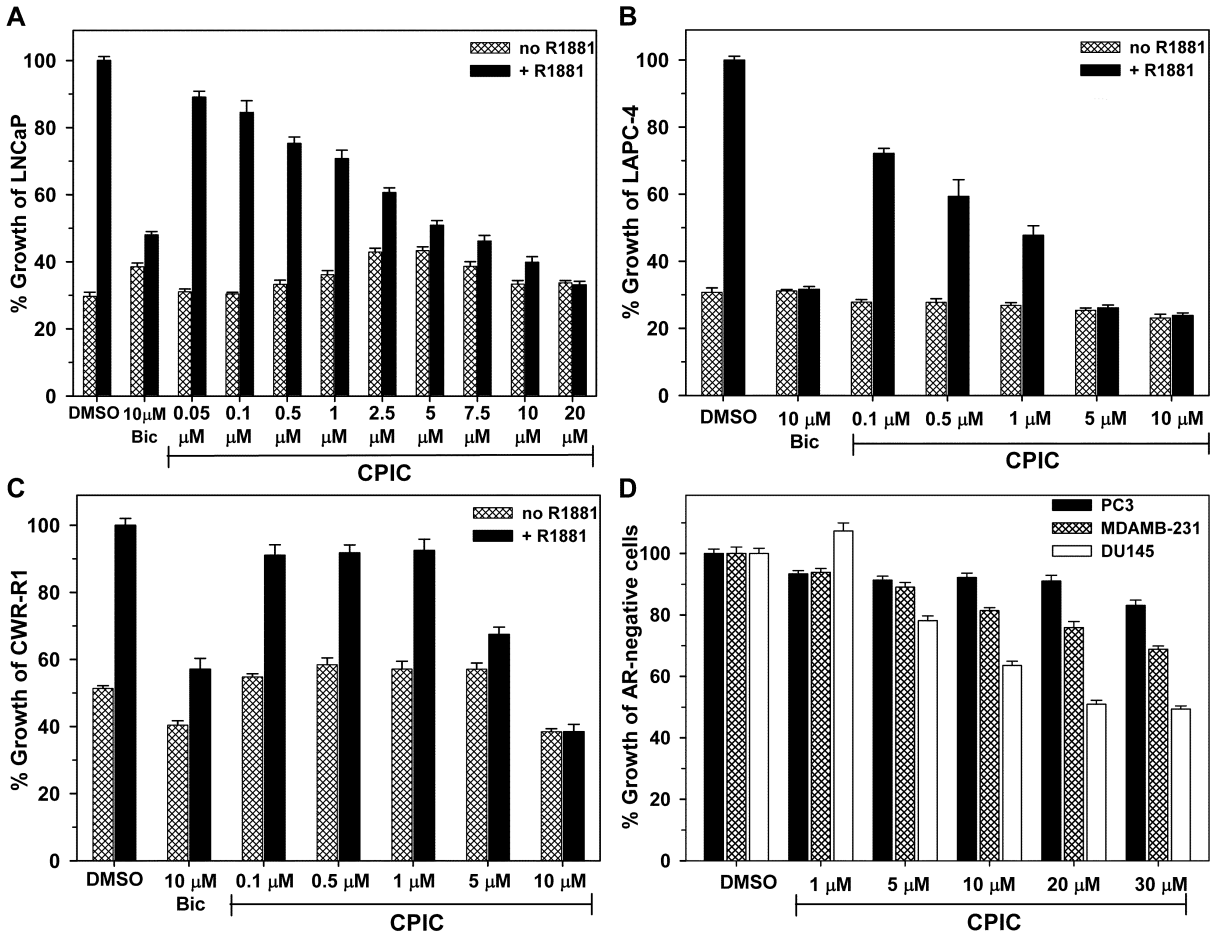



FIGURE 4.5. **Effects of CPIC on prostate cancer cell growth.** *A*, LNCaP cells, *B*, LAPC-4 cells, and *C*, CWR-R1 cells were treated with the indicated concentrations of CPIC in the presence or absence of 1 nM R1881. After 4 days of treatment for LNCaP and CWR-R1 cells, and 8 days for slow-growing LAPC-4, cell proliferation was measured using MTS. Cell growth in R1881+DMSO was set to 100%. *D*, AR-negative PC-3, MDA-MB-231 and DU145 cells were inoculated at 2,000 cells/well in 96-well plates and treated with 100 µl medium containing the indicated concentrations of CPIC or DMSO (vehicle). Growth of the cells was evaluated after 3 days. DMSO wells were set to 100%. Data points represent the mean of 8 wells ± SEM.

**Figure 4.6**

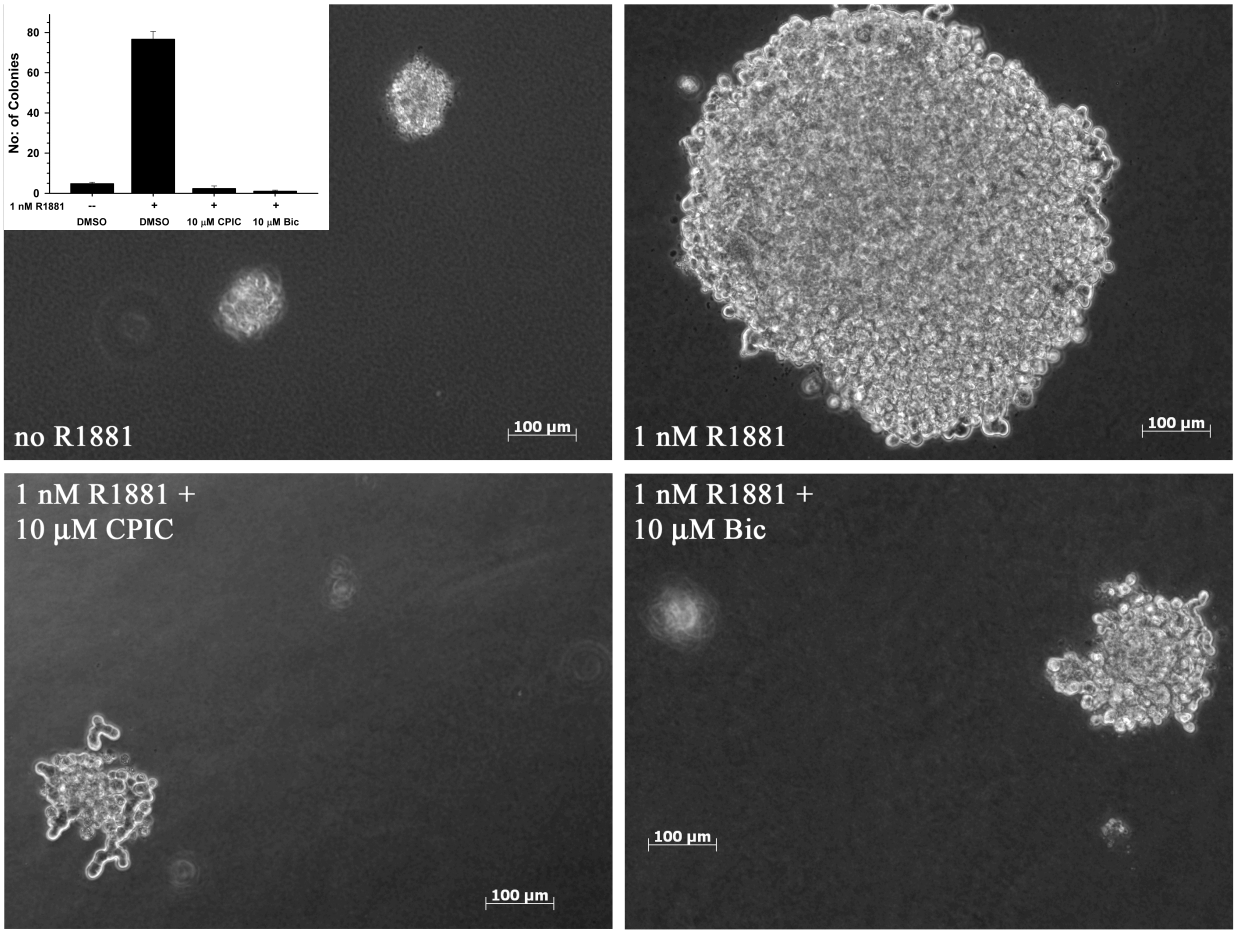



FIGURE 4.6. **CPIC inhibits anchorage-independent growth of LAPC-4 prostate cancer cells in soft agar.** 5,000 LAPC-4 cells were plated into top agar. Cells were treated with medium containing DMSO (vehicle), 1 nM R1881, 1 nM R1881 + 10 μM CPIC or 1 nM R1881 + 10 μM Bic, and replenished every 3-4 days. After 28 days colonies were counted and photographed at 5X magnification. *Inset*, Bar graph represents the average of the total number of colonies counted in each well of the treatments. Photographs are representative of the entire well and of triplicate experiments.

**Figure 4.7**

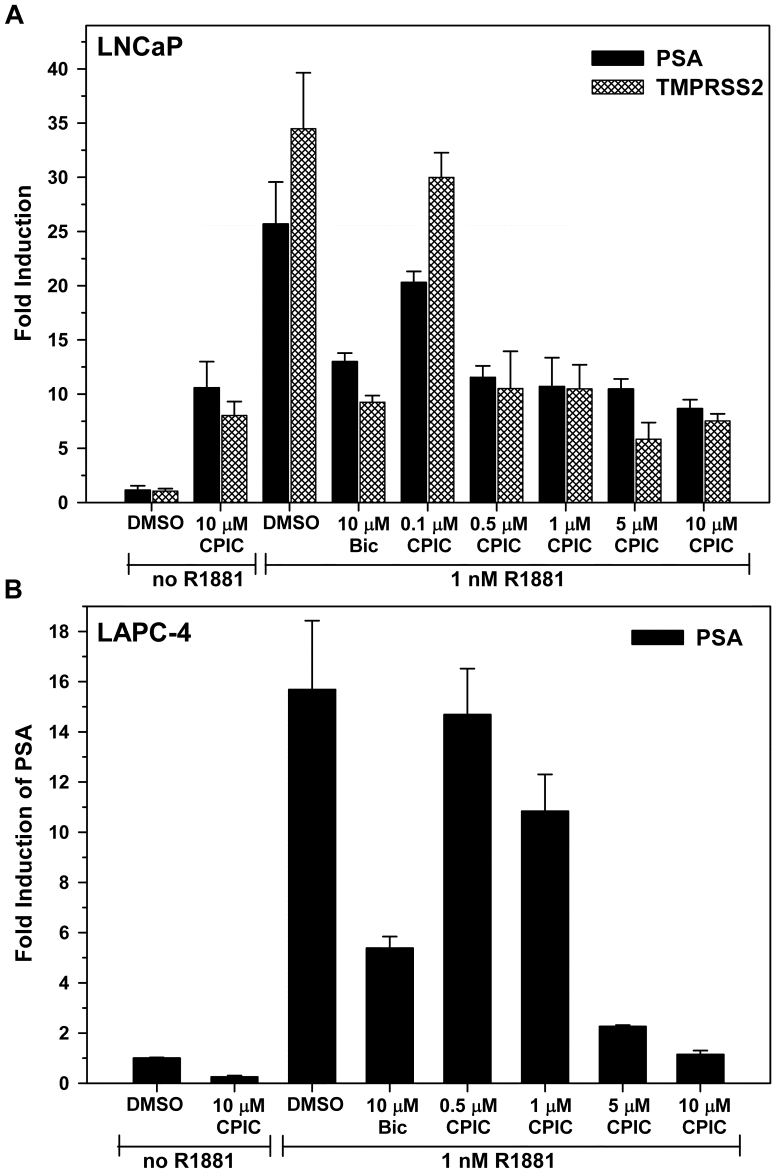



FIGURE 4.7. **CPIC inhibits expression of endogenous AR-regulated genes.** *A*, LNCaP and *B*, LAPC-4 cells were seeded in 6-well plates at 300,000 cells/well in medium containing 5% CD-FBS for at least 3 days. Cells were treated with or without 1 nM R1881 and the indicated concentrations of CPIC or bicalutamide (Bic) for 24 h before RNA extraction. mRNA was quantitated using qRT-PCR and normalized to β-actin. Data represent the mean of 3 independent experiments ±SEM.

**Figure 4.8**

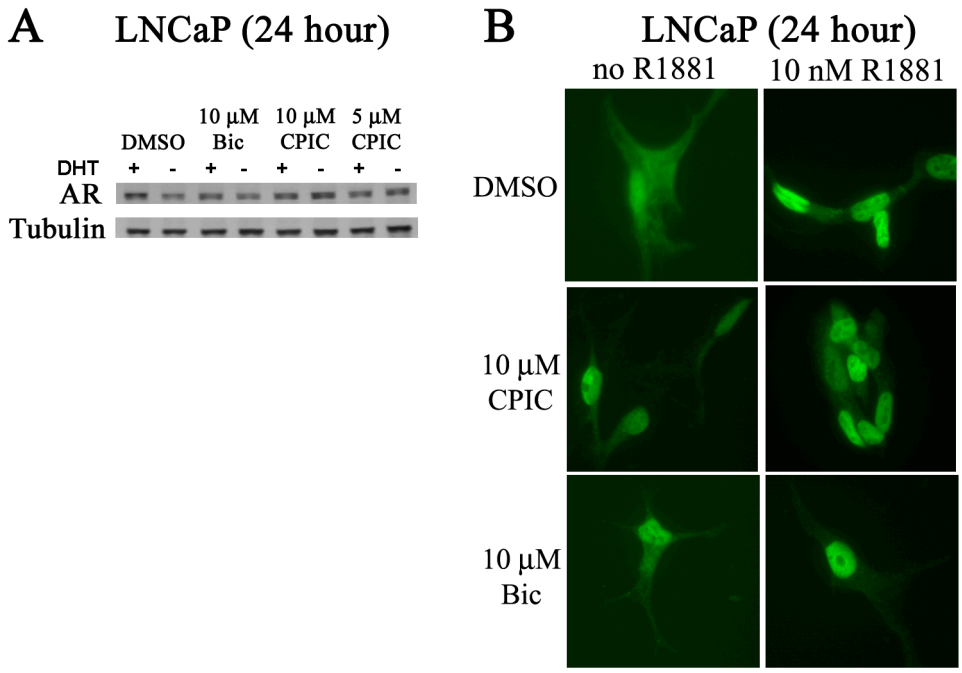



FIGURE 4.8. **CPIC does not decrease AR levels or alter nuclear localization of AR.** *A*, Western blot of AR levels in LNCaP cells treated with CPIC. LNCaP cells were plated in medium containing 5% CD-FBS and maintained for at least 3 days. Treatment medium with (+) or without (-) 10 nM DHT containing DMSO (vehicle) or the indicated inhibitor was added to the cells. After 24 h, cell lysates were prepared and equal amounts of protein were analyzed by western blotting and tubulin used as a loading control. *B*, Visualization of intracellular AR in LNCaP cells. LNCaP cells were incubated with 10 nM R1881, 10 µM Bic, or 10 µM CPIC for 24 h and intracellular AR was visualized by fluorescent microscopy using AR polyclonal antibody (Abcam, ab3510). Objective magnification, 40X.
Experimental Data in Figure 4.8B: EM Wilson

**Figure 4.9**

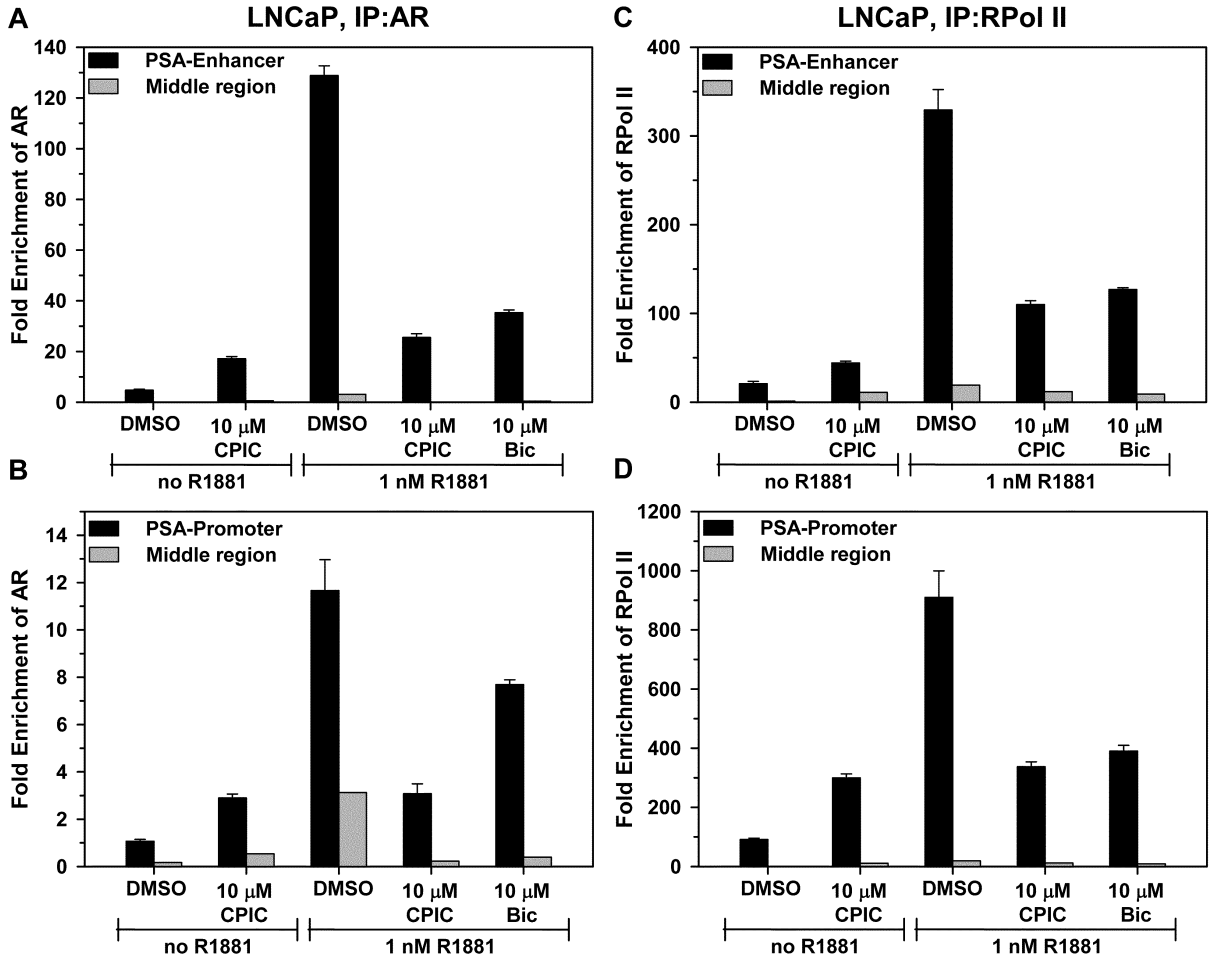



FIGURE 4.9. **CPIC inhibits R1881-AR occupancy at AREs in LNCaP cells.** Prior to ChIP, LNCaP cells were maintained for 3 days in medium containing 5% CD-FBS. After 1 h incubation with DMSO, 10 μM CPIC or 10 μM Bic, the cells were treated with 1 nM R1881 or ethanol and incubated for 4 h. Protein complexes were cross-linked and AR (*A*) or RPol II (*B*) or control IgG antibody was used to pull-down protein bound chromatin fragments. Occupancy at the PSA-enhancer, PSA-promoter and a control ARE-free region (middle region) between the two sites was determined using qRT-PCR. Fold enrichment over IgG control was plotted. Data represent the mean of 3 PCRs ± SEM and are representative of other experiments. Significance of the differences between CPIC and the DMSO control was tested using Student's T-test. $p<0.01$ when compared to the respective controls.

**Figure 4.10**

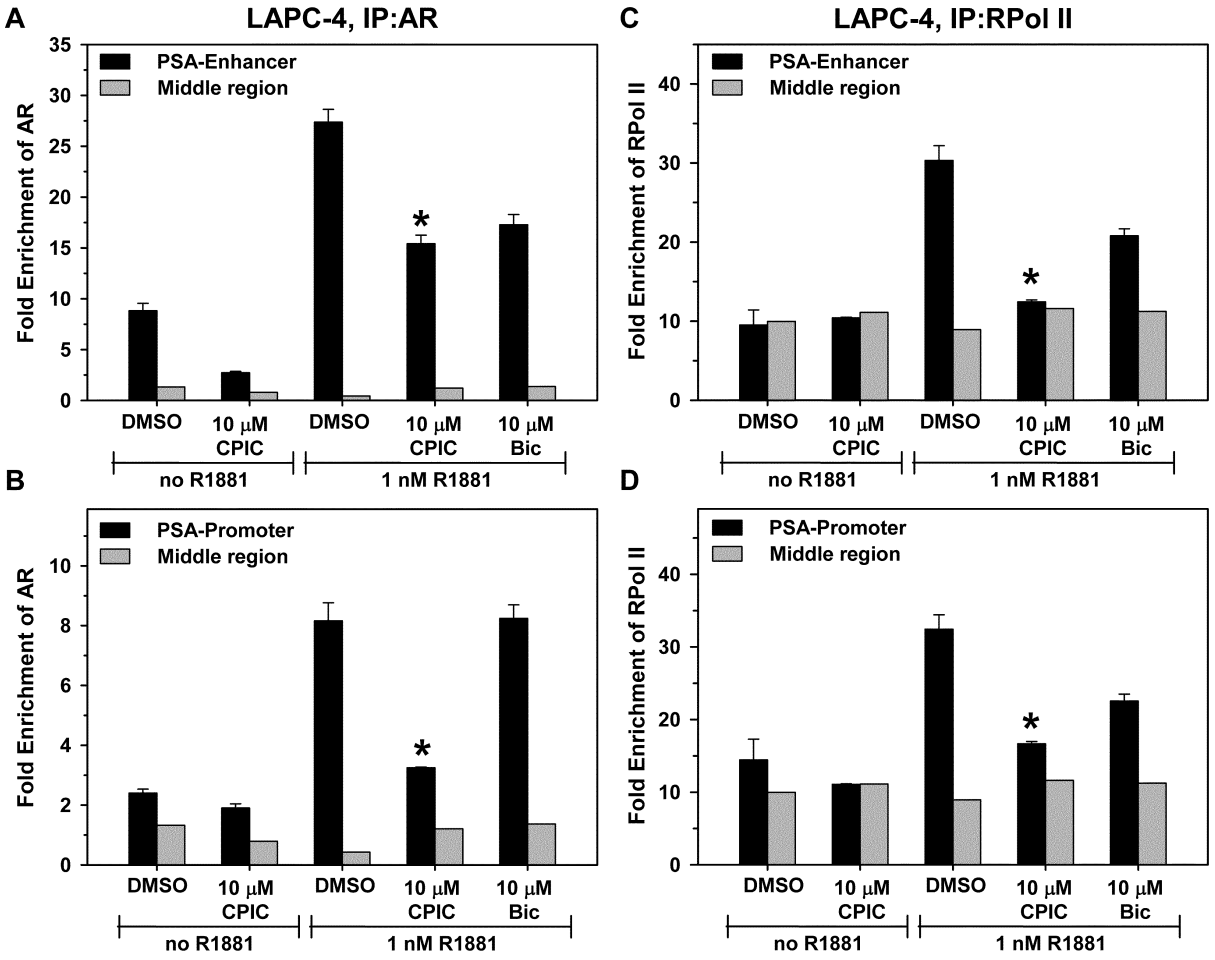
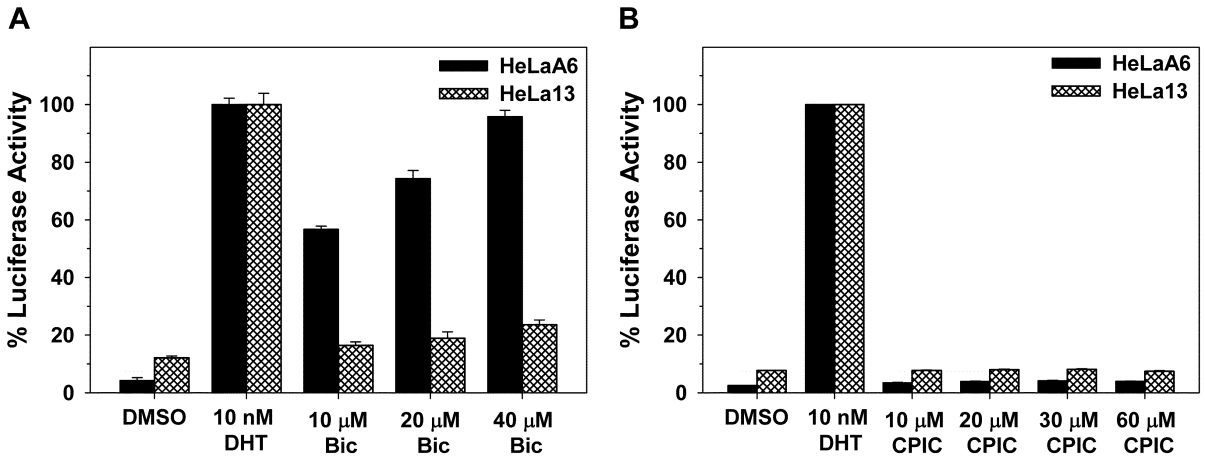



FIGURE 4.10. **CPIC inhibits R1881-AR occupancy at AREs in LAPC-4 cells.** LAPC-4 cells were maintained for 3 days in medium containing 5% CD-FBS and ChIP was carried out as described in Fig. 4.9 and Experimental Procedures. AR (*A, B*) and RPol II (*C, D*) occupancy at the PSA-enhancer, PSA-promoter and a control ARE-free region (middle region) between the two sites were determined using qRT-PCR. Fold enrichment over IgG control was plotted. Data represent the mean of 3 PCRs ± SEM. Significance of the differences between CPIC and the DMSO control was tested using Student's T-test and $p < 0.05$ when compared to the respective controls. For AR recruitment to the PSA-promoter, R1881+10 μM Bic was not significantly different from R1881+DMSO.

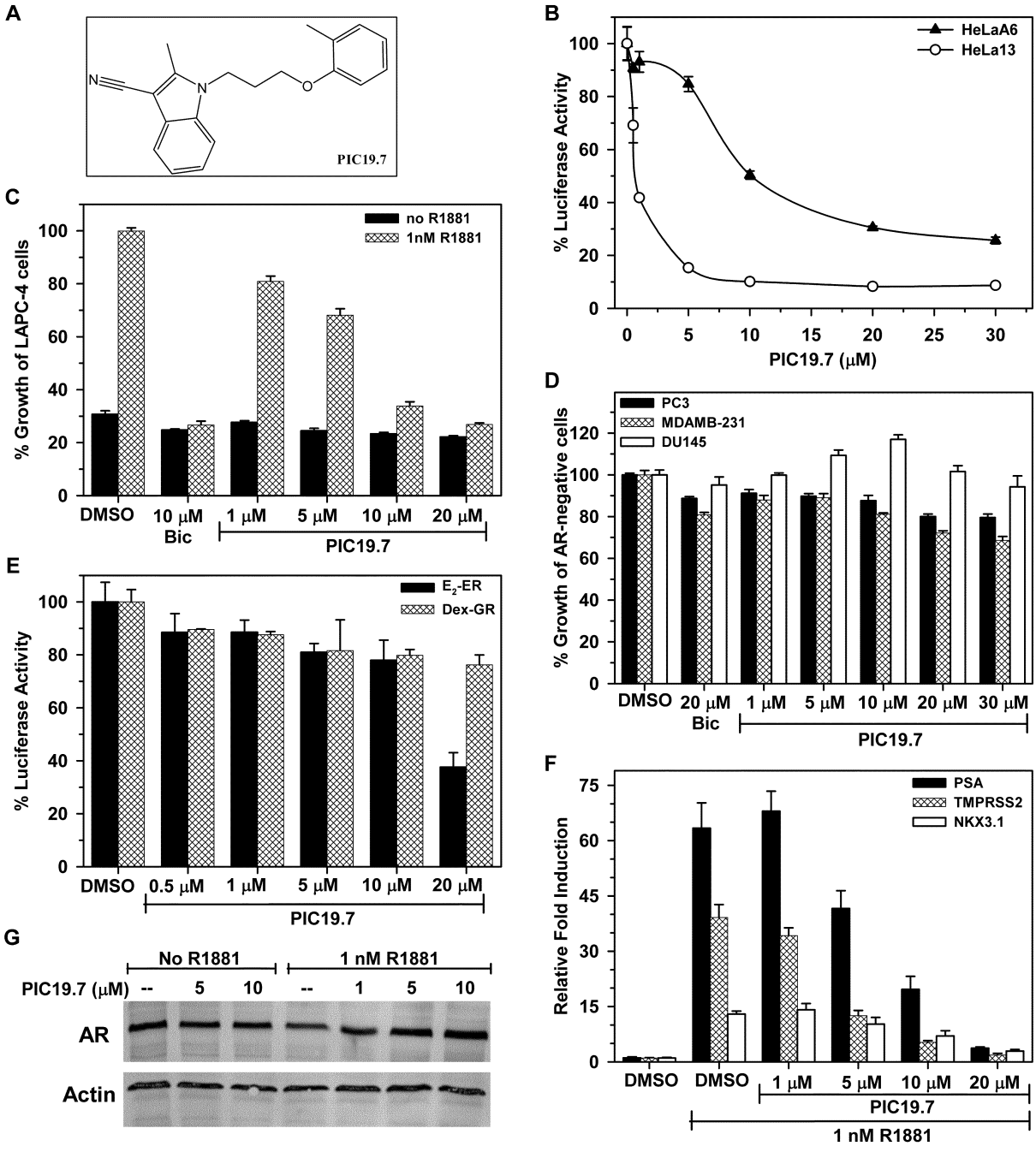
## SUPPLEMENTAL FIGURES

### Supplemental Figure 4S.1




SUPPLEMENTAL FIGURE 4S.1. **Bicalutamide is an agonist in HeLaA6 cells but not CPIC.** Dose-response studies of (*A*) bicalutamide and (*B*) CPIC activation of AR-induced PSA-luciferase in HeLaA6 and HeLa13 cells in the absence of added androgen. The cells were seeded in 24-well plates and treated with medium containing DMSO (vehicle), 10 nM DHT, or the indicated concentrations of Bic or CPIC for 24 h and assayed for luciferase activity. Activity of the reporter in the presence of 10 nM DHT was set to 100%. Data represent the mean of 3 experiments ±SEM.

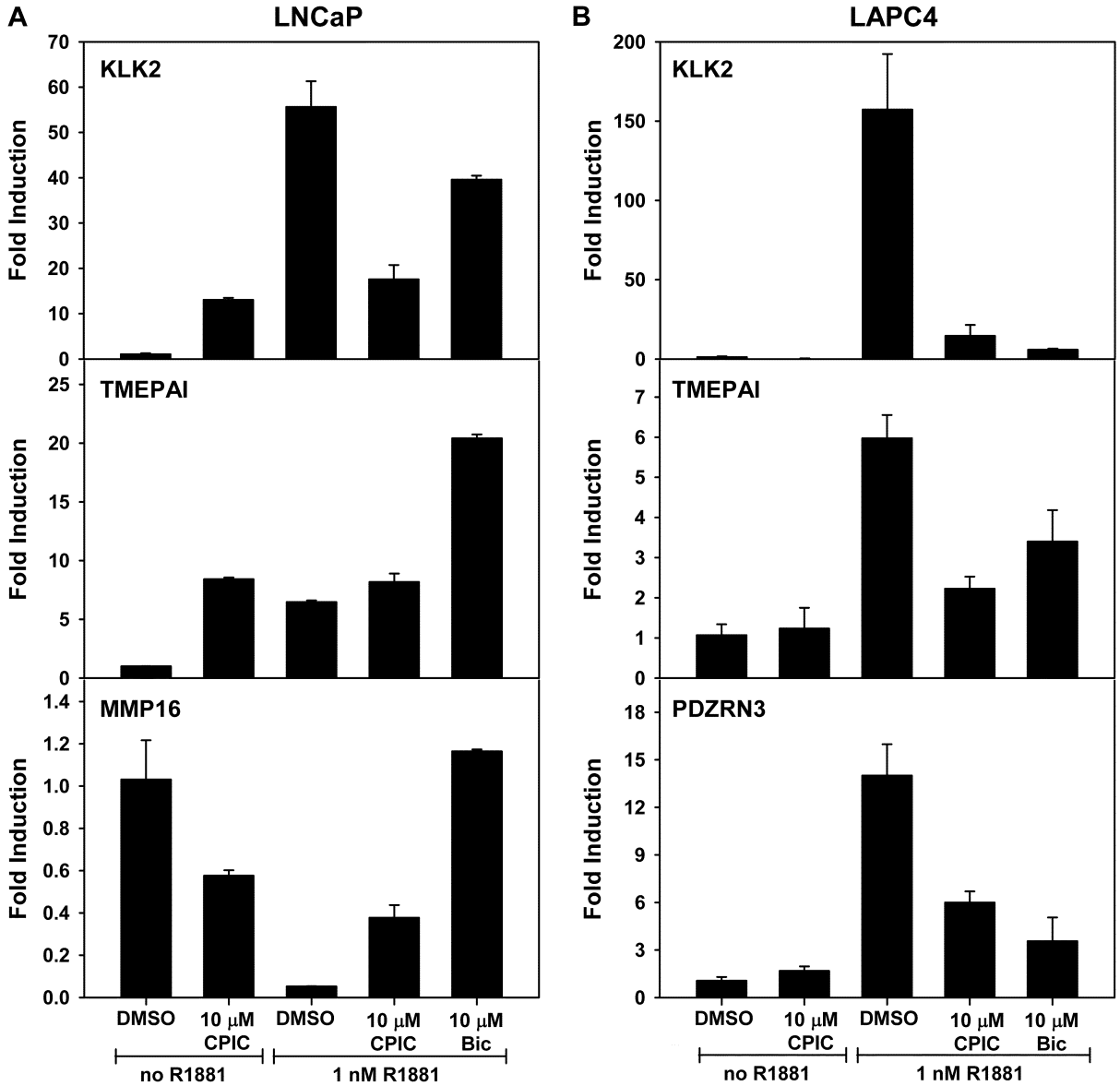
**Supplemental Figure 4S.2**




SUPPLEMENTAL FIGURE 4S.2. **PIC19.7 is a less potent analogue of CPIC.** *A*, Structure of PIC19.7. *B*, PIC19.7 inhibits androgen-mediated luciferase activity in androgen-treated HeLaA6

and HeLa13 cells. *C* and *D*, PIC19.7 inhibits androgen-dependent growth of LAPC-4 cells without exerting toxic effects on the AR-negative PC3, MDA-MB-231 and DU145 cells. *E*, PIC19.7 is a specific inhibitor of AR and has minimal effects on GR or ERα induced luciferase activity. *F*, PIC19.7 inhibits AR mediated gene expression in LNCaP cells. *G*, In LNCaP cells, AR protein is not degraded in the presence of PIC19.7.

**Supplemental Figure 4S.3**

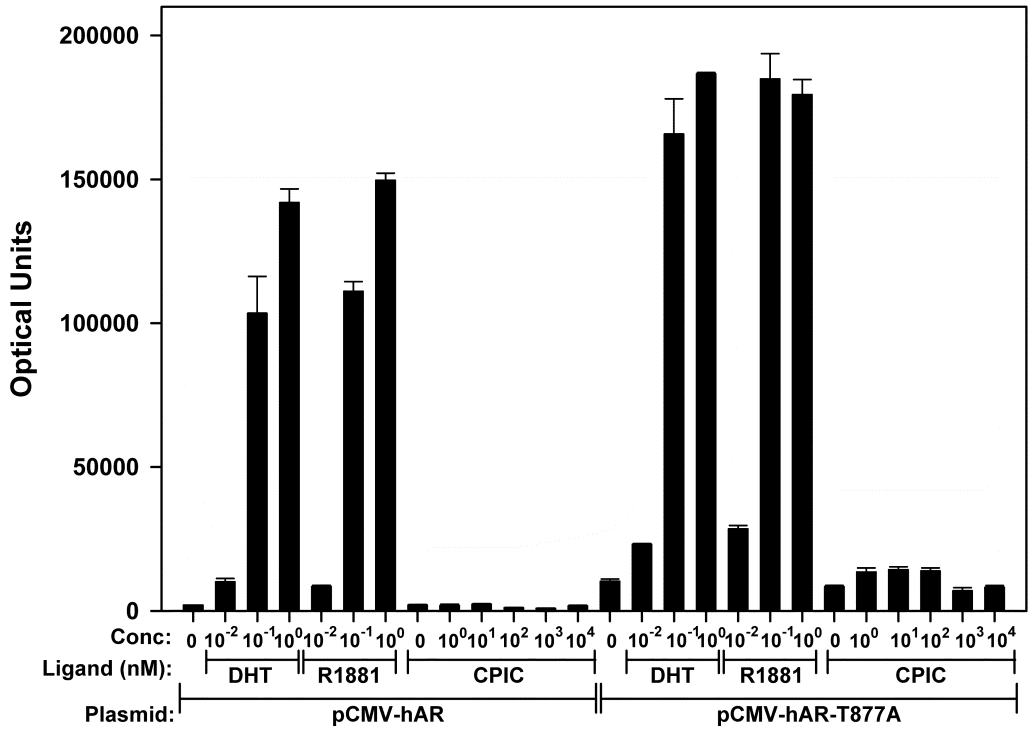



SUPPLEMENTAL FIGURE 4S.3. **Effect of CPIC on androgen-regulated gene expression.**

*(A*) LNCaP or *(B)* LAPC-4 cells were treated with or without 1nM R1881 and DMSO (vehicle), or 10 μM CPIC, or bicalutamide (Bic) for 24 h. qRT-PCR was performed to measure the levels of several known androgen-regulated mRNAs. Levels of mRNA were normalized to levels of *β-actin* mRNA and presented as a ratio to control wells (no R1881, DMSO). Bars represent the mean of 3 experiments ± SEM.

Primers used were: KLK2 forward primer 5'- TGT GTG CTA GAG CTT ACT CTG and reverse primer 5'- CCA CTT CCG GTA ATG CAC CA; TMEPAI forward primer 5'- AAC GCT CTT TGT TCC AGA GCA TGG and reverse primer 5'- TCA CCA CCA CCA TCA CCA TCA TCA; PDZRN3 forward primer 5'- AAT GGG ATA GAG GTG CAG AAC CGT and reverse primer 5'- ATG TCC ATG TGC AGG TCA TCC AGA; MMP16 forward primer 5'- TCT CCT CAG GGA GCA TTT GT and reverse primer 5'- TCC TTG AGG ATG GAT CTT GG.

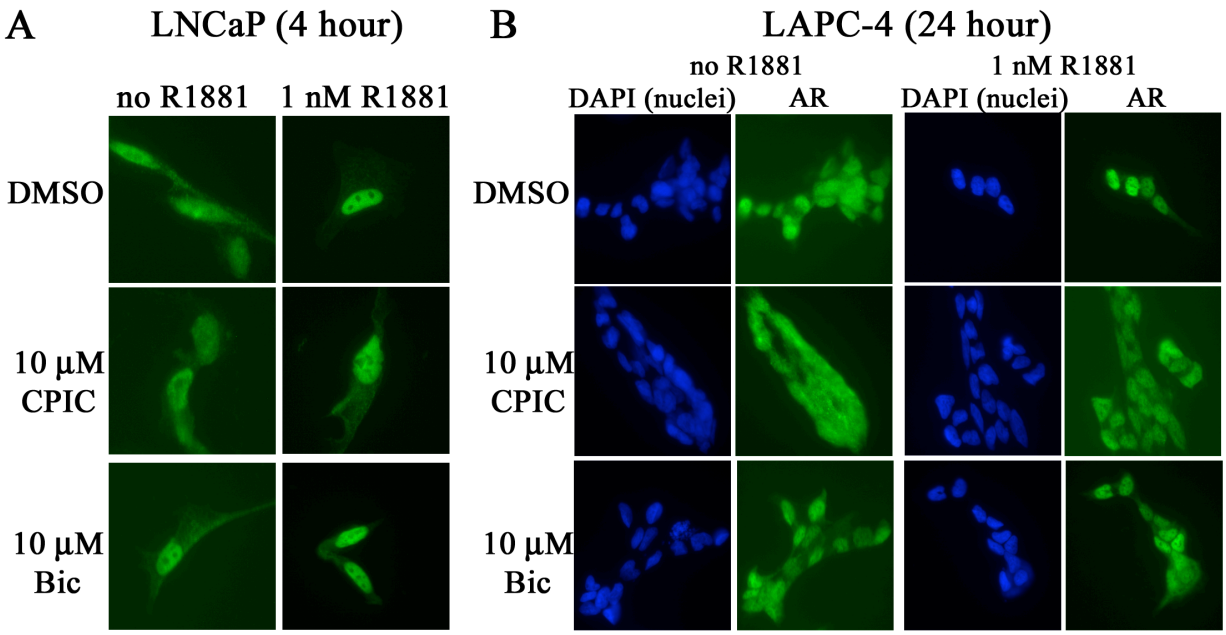
**Supplemental Figure 4S.4**




SUPPLEMENTAL FIGURE 4S.4. **CPIC does not exhibit partial agonist activity in HeLa cells transfected with AR-T877A mutant or wt-AR.** HeLa cells were transiently transfected with pCMV-hAR or pCMV-AR-T877A and PSA-luciferase reporter plasmids (as described in (29)), and treated with indicated nM concentrations of ligands DHT, R1881 or CPIC. The average of duplicate experiments is shown.

Experimental Data in Supplemental Figure 4S.4: EM Wilson

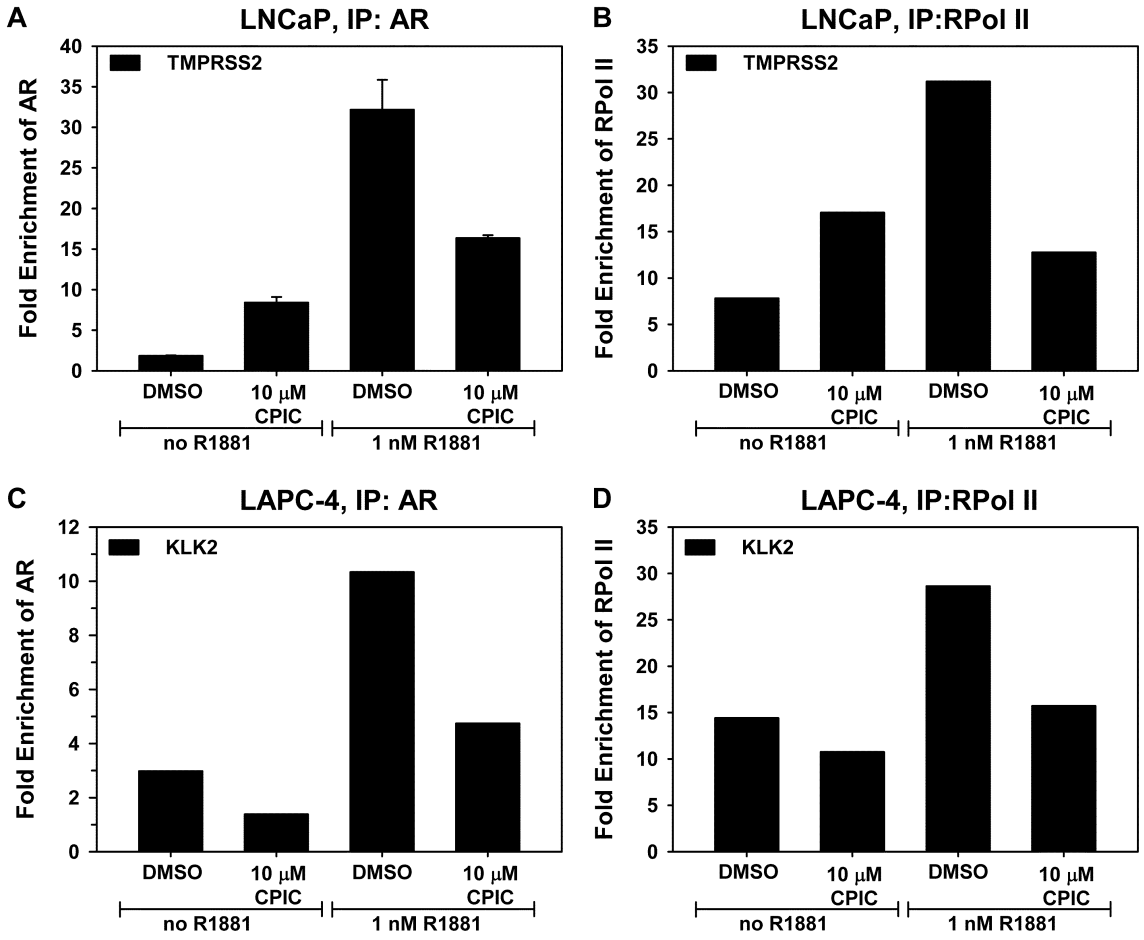
**Supplemental Figure 4S.5**




SUPPLEMENTAL FIGURE 4S.5. **CPIC does not alter nuclear translocation of AR in LNCaP or LAPC-4 cells.** *(A)* LNCaP or *(B)* LAPC-4 cells were incubated with 1 nM R1881, 10 µM Bic, or 10 µM CPIC for 4 or 24 h, respectively. AR was visualized by fluorescent microscopy using AR polyclonal antibody (Abcam, ab3510). Intracellular DNA was visualized by DAPI staining. Objective magnification, 40X.

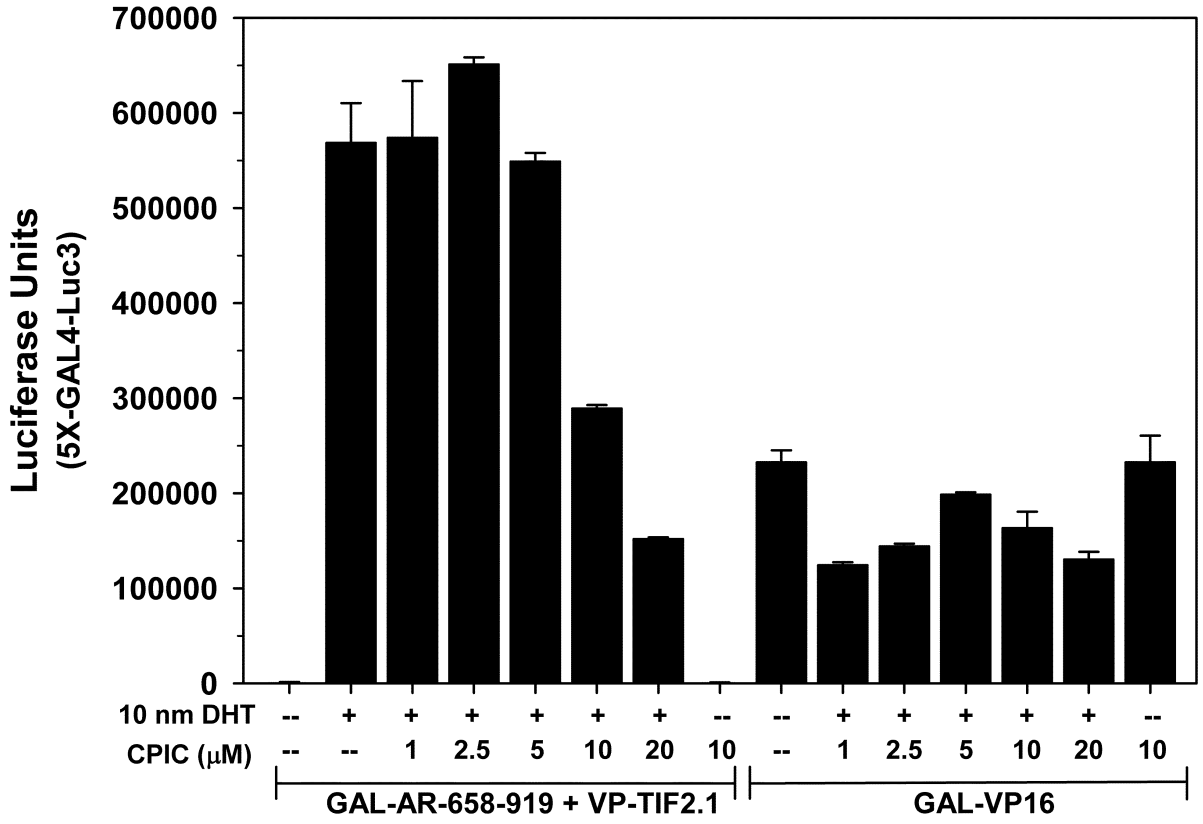
Experimental Data in Supplemental Figure 4S.5: EM Wilson

**Supplemental Figure 4S.6**




SUPPLEMENTAL FIGURE 4S.6. **CPIC inhibits AR and RNA polymerase II (RPol II) occupancy at TMPRSS2 and KLK2 genes in LNCaP and LAPC-4 cells.** LNCaP and LAPC-4 cells were maintained for at least 3 days in 5% CD-FBS medium prior to ChIP. After one hour incubation with DMSO or 10 μM CPIC, cells were treated with 1 nM R1881 or ethanol and further incubated for 4 h. Protein complexes were cross-linked and *(A,C)* AR antibody, *(B,D)* RPol II antibody or control normal mouse IgG was used to pull-down protein bound chromatin fragments. Occupancy at the TMPRSS2-enhancer site in LNCaP cells and KLK2-enhancer site in LAPC-4 cells was determined using qRT-PCR. Fold enrichment over IgG control was plotted. Primers used were: KLK2 forward primer 5'- GTT GAA AGC AGA CCT ACT CTG GA and reverse primer 5'- CTG GAC CAT CTT TTC AAG CAT; TMPRSS2-Enh-ARE forward primer 5'- TGG TCC TGG ATG ATA AAA AAA GTT T and reverse primer 5'- GAC ATA CGC CCC ACA ACA GA

**Supplemental Figure 4S.7**




SUPPLEMENTAL FIGURE 4S.7. **High concentrations of CPIC interrupt TIF2 interaction with AR-LBD in two-hybrid assays.** HeLa cells were plated in 12-well plates at 50,000 cells/well and transfected with 100 ng 5XGAL4Luc3 reporter plasmid, and either 50 ng GAL-AR-658-919 and 50 ng VP-TIF2.1 or 50 ng GAL-VP16 control plasmid. Cells were incubated in the presence or absence of 10 nM DHT and the indicated concentrations of CPIC. The mean and S.E. of luciferase activity are representative of two independent experiments.

Experimental Data in Supplemental Figure 4S.7: Elizabeth M Wilson

*Plasmids*: VP-TIF2.1 coding for TIF2 residues 624–1287 as a fusion protein with the VP16 activation domain was generously provided by Heinrich Gronemeyer (Institute of Genetics and Molecular and Cellular Biology) and the GAL4 luciferase reporter 5XGAL4Luc3 containing five tandem GAL-4 binding sites and the luciferase coding sequence was from Donald P. McDonnell (Duke University).

# CHAPTER 5
# CPIC INHIBITS TRANSACTIVATION BY THE AMINO-TERMINAL DOMAIN OF THE ANDROGEN RECEPTOR

The androgen receptor (AR) is unusual in the critical role of its N-terminal domain (NTD). While the C-terminal ligand-binding domain is the primary site for coactivator binding in other steroid receptors, the N-terminal domain plays a key role in coactivator binding to AR. Also, AR action is dependent on a strong interdomain N/C interaction. Our earlier work (see Chapter 4) suggested that CPIC was extremely effective in inhibiting the AR N/C interaction. We therefore looked in more detail at the ability of CPIC to inhibit the AR N/C interaction and its ability to inhibit transactivation by a constitutively active AR fragment that contains the N-terminal domain and the DNA binding domain but lacks the ligand binding domain. CPIC, potently inhibited transactivation by truncated AR. In contrast, the classical ligand binding competitors, bicalutamide and hydroxyflutamide had no effect on transactivation by truncated AR. CPIC also inhibited androgen-independent proliferation of CWR22RV1 cells, in which proliferation is stimulated by an AR splice variant that lacks the ligand-binding domain. CPIC shows a competitive ligand phenotype but is also able to act at the N-terminus. CPIC reduces the AR N/C interaction, presumably by interacting with the AR-NTD. We hypothesize that CPIC binding disallows androgens from binding the receptor at the LBD. CPIC could bind near the N-terminus to stabilize an inactive AR conformation that opposes ligand binding. There is a slight possibility that CPIC binds at two sites on AR, one in the ligand binding pocket and one in the N-terminal region. Alternatively, it remains to be seen if CPIC inhibits some unknown external factors (like cytokines, kinases or growth factors), the activity of which might be crucial for AR transactivation in most cell types. CPIC represents a class of compounds with properties that make them suitable candidates for further therapeutic development in CRPC.

## INTRODUCTION

Androgens, such as dihydrotestoserone, and the synthetic androgen R1881, exert their actions by binding to the androgen receptor (AR). AR has modular functional domains with an amino-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region, and a C-terminal ligand-binding domain (LBD). Non-liganded full-length AR is found associated with a complex

of molecular chaperones in the cytoplasm. Binding of androgen to the LBD of the AR induces an intramolecular conformation change in the receptor that brings the amino and carboxy termini into close proximity, resulting in an N/C terminal interaction (1). Activated AR translocates and accumulates in the nucleus, where it can effectively bind to specific androgen response elements (AREs) in the promoter and enhancer regions of androgen-regulated genes to initiate transcription. The intra-domain N/C terminal interaction within the receptor and interactions of each of the domains with coactivators, corepressors, and DNA are essential for the regulation of transcription elicited by androgen-bound AR at responsive genes. The N-terminal region of AR contains the major activation domains (AF1 and AF5). Even in the absence of androgen, AR can also be activated by signaling pathways acting through the N-terminal region via c-AMP activated kinase (PKA), IL-6 or bone-derived factors (2).

AR plays a key role in the growth and progression of prostate cancer (2-4). After primary treatment by surgical removal of the tumor or radiation, about 1/3rd of prostate tumors will eventually recur and are highly malignant (Gleason Score ≥7). These tumors are currently treated using endocrine therapies that either block production of androgens (surgically or chemically) or using anti-androgens such as Casodex (bicalutamide), hydroxyflutamide and the experimental drug MDV3100 that act by competing with androgens for binding to androgen receptor. Usually the tumor will begin to regrow in the absence of testicular androgens and in spite of anti-androgen treatment. These tumors are called castration recurrent (or castration resistant) prostate cancer (CRPC). Therapeutic options for CRPC patients are very limited.

It is widely accepted that CRPC remains dependent on AR action. Prostate specific antigen (PSA) and other androgen-AR induced genes which are detected in primary prostatic tumors are also elevated in CRPC (5). AR is the most commonly overexpressed protein in CRPC (6). Generation of gain of function mutations in the receptor, and altered action of coregulatory proteins, increased cross talk with other signaling pathways or the action of constitutively active AR splice variants can all contribute to disease progression in CRPC (7).

The amino (N)-terminal region of the androgen receptor plays an important role in its ability to regulate gene expression. A regulatory protein, called MAGE-11 is overexpressed in many prostate cancers (8), and activates androgen receptor activity by binding to the N-terminal region. Also, some prostate cancers contain short forms of the androgen receptor protein that contain the N-terminal region and lack the part of the AR that binds androgens but continue to be

transcriptionally active. Because they are missing the site that binds androgens, they do not respond to agents that act by competing with androgens for binding to the androgen receptor. Recently, naturally occurring splice variants containing only the N-terminal region of AR and the DNA binding domain and some C-terminal extensions have been identified in CRPC (9-12). These constitutively active variants of AR retain transcriptional activity similar to that of full-length receptor. Since they lack the binding site for androgens, they are not activated by androgens and are insensitive to inhibition by anti-androgens that compete with androgens for binding to the AR, such as bicalutamide and hydroxyflutamide. The AR NTD, with its high intrinsic disorder and flexibility, has not yet been crystallized and has traditionally not been targeted for structure-based drug design. But targeting the AR NTD would allow us to block in vivo prostate tumor growth in the presence of androgens in androgen-sensitive tumors and in the absence of androgens as seen in CRPC.

Most recent inhibitors of prostate cancer progression have targeted activation of the AR, either directly or through co-regulators, or concentrated on the reduction of androgen synthesis by inhibiting 17,20-lyase/CYP17 activity. Of the androgen synthesis inhibitors, abiraterone acetate (Zytiga) has been approved for clinical use in the treatment of hormone-refractory prostate cancer while TOK-001 and TAK-700 (13, 14) are in various stages of clinical trials. New investigational drugs MDV3100 and a close analogue ARN-509 are antiandrogens that have shown great promise in clinical trials of CRPC. Current therapeutics bicalutamide and hydroxyflutamide (OHF) demonstrate agonist properties in cells containing mutant AR or expressing high AR levels (15, 16).

To identify small molecules that inhibit the growth of cancer cells that contain androgen receptor, we carried out high throughput screening (HTS). We used the ~160,000 compounds in the libraries in the UIUC High Throughput Screening Center and carried out a cell-based assay measuring the ability of the small molecules to inhibit androgen-AR-induced expression of an easily assayed “reporter gene” consisting of a regulatory region controlling expression of PSA, driving production of the easy to assay luciferase. Serum PSA is a widely used diagnostic and prognostic indicator in prostate cancer. Small molecule “hits” were then validated by re-testing, evaluated for potency (the amount of the small molecule required to produce an effect), for efficacy (the maximum effect seen) and for specificity as an inhibitor of AR and not the closely related steroid hormone receptor, the estrogen receptor (ER) and for the absence of toxicity in

cells that do not contain AR. Then verified hits were tested for the ability to inhibit proliferation of AR-containing prostate cancer cells with low toxicity to AR-negative cells. By far the most effective compound to emerge from the screen and follow-up studies was CPIC (1-(3-(2-chlorophenoxy)propyl)-1H-indole-3-carbonitrile) as described previously in Chapter 4. CPIC is able to:

(i) Inhibit androgen-AR action, even in cells in which bicalutamide/casodex is ineffective

(ii) Block the induction of endogenous androgen regulated genes (TMPRSS2, PSA and others) in multiple cell lines

(iii) CPIC acts primarily by inhibiting binding of androgen-AR to regulatory regions of responsive genes

(iv) Inhibit anchorage-dependent and anchorage-independent androgen-stimulated growth of prostate cancer cells with little effect on AR-negative cell lines

(v) At 0.5 μM CPIC, completely inhibits the AR N/C interaction

Here we describe CPIC as a small molecule inhibitor of androgen receptor that is effective against both the full-length androgen receptor and against a short truncated androgen receptor that does not respond to current drugs. CPIC completely inhibits constitutively active truncated AR1-660 that lacks the ligand-binding domain at concentrations as low as 0.5 μM. CPIC blocks androgen-dependent and androgen-independent growth of CWR22Rv1 prostate cancer cells. The sub-micromolar potency of CPIC against truncated AR is especially impressive compared to other small molecules that target AR (17). CPIC exhibits a wide therapeutic window. CPIC represents a class of compounds with properties that make them suitable candidates for further therapeutic development in CRPC.

## MATERIALS & METHODS

***Plasmids***: Expression vectors used have been previously described (18). pCMV-AR-(507–919) codes for the human AR DNA and ligand-binding domains, pCMV-AR-(1–503) codes for the AR amino-terminal domain. PSA-Enh-Luc containing the PSA upstream enhancer region was generously provided by Michael Carey (University of California, Los Angeles). The pCMV-AR-1-660 codes for the AR amino terminal domain and DBD with parts of the hinge region (Exons 1/2/3) and is constitutively active in the absence of androgens. pCMV-hAR and pCMV-hGR express full-length androgen and glucocorticoid receptors, respectively, and binds to the

PSA-Enh-Luc to induce luciferase activity in the presence of their respective hormone ligands dihydrotestosterone (DHT) or dexamethasone (Dex).

Expression plasmids for a chimeric protein encoding the GAL4 DNA binding domain fused to the C-terminal AR ligand binding domain (GAL -AR-658-919) and the N-terminal region of AR fused to the powerful VP16 transcription activation domain (VP-AR-1-660) interact with each other and induces luciferase enzyme activity through the 5X-GAL4 DNA element, in the presence of androgens. The GAL-VP16 plasmid is used as a control.

***Transient transfections***: Transfections in HeLa cells were performed as described (19). Briefly, human epithelial cervical carcinoma HeLa cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and 2 mM-glutamine. HeLa cells were plated at $5 \times 10^4$ cells/well in 12-well plates and 24 h later transfected using FuGENE 6 transfection reagent (Roche Applied Science) with indicated amounts of expression vectors and luciferase reporter plasmid to determine androgen-induced or androgen-independent AR transactivation. The next day cells were washed with PBS, and 1 ml/well serum-free medium containing the indicated treatments was added and incubated at 37 °C. Twenty four hours later cells were washed with PBS and assayed for luciferase activity after harvesting in 0.25 ml of lysis buffer using a Lumistar Galaxy (BMG Labtech) automated multi-well plate reader luminometer.

***Cell proliferation assay***: CWR22Rv1 cells were obtained from ATCC and maintained in RPMI 1640 supplemented with 10% FBS and 1% penicillin and streptomycin at 37˚C in 5% $CO_2$. 4 days prior to experiments the cells were shifted to media containing 5% CD-treated FBS for removal of any trace androgens. 2,000 cells/well were plated in 96-well plates and allowed to plate for a day. Treatment medium containing indicated concentrations of inhibitor compounds was added to the cells, with or without 1 nM DHT and incubated for 6 days. Cell viability was determined using Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS).

## RESULTS

### *CPIC disrupts the AR N/C interaction*

Interaction between the amino (N) terminal region of AR and the ligand-binding domain (C-terminal region) plays a critical role in AR activity. To evaluate the effect of CPIC on the interdomain AR N/C interaction, we performed mammalian 2-hybrid assays using constructs

containing the N- and C-terminal regions of AR. Transfections were performed in HeLa cells using pCMV-AR-(1-503) (the AR $NH_2$-terminal region), pCMV-AR-(507-919) ($NH_2$-terminal deletion of AR) and PSA-Enh-Luc reporter plasmids in the absence and presence of 10 nM DHT. Bicalutamide and other antagonists that bind to the ligand-binding pocket of AR disrupt this crucial interaction and alter receptor structure and function at higher concentrations (1, 20). CPIC was substantially more effective than bicalutamide in inhibiting the androgen-induced AR N/C interdomain interaction (Fig. 5.1).

CPIC is able to reduce the recruitment of AR to DNA regulatory sites on target genes (Fig 4.9 and 4.10). To eliminate the possibility that the reduction we see in luciferase activity in the above assay is due to a poor recruitment of the interacting AR fragments to the PSA-enhancer-luciferase, we tested the ability of CPIC to disrupt N/C interaction by using GAL4-VP16 two-hybrid constructs. In these constructs, GAL4-DBD not AR-DBD is responsible for DNA binding. Expression plasmids for a chimeric protein encoding the Gal4 DNA binding domain fused to the C-terminal AR ligand binding domain (Gal-AR-658-919) and the N-terminal region of AR fused to the powerful VP16 transcription activation domain (VP-AR-1-660) were used. Treatment with 10 nM DHT results in robust transcription of the luciferase reporter. CPIC elicited a dose-dependent inhibition of the reporter with an $IC_{50}$ of ~0.7 μM and complete inhibition at 10 μM (Fig. 5.2). CPIC inhibition was specific for the AR N/C interaction. Control transfections with GAL-VP16, which activates the same reporter, showed no effect of CPIC at all concentrations tested (0.1-10 μM). These data show that CPIC potently inhibits the N/C interaction of AR.

***CPIC potently inhibits transcription by the truncated N-terminal AR***

Truncated AR variants that contain the NTD and DBD but lack the LBD are constitutively active in the absence of androgens. Dose-response studies in transfected cells showed that CPIC potently inhibited AR1-660 constitutive activity, with $IC_{50}$ = ~0.2 μM and >90% inhibition at 0.5 μM (Fig. 5.3). AR1-660 lacks the AR LBD and does not bind androgens, and thus bicalutamide (Bic) and hydroxyflutamide (OHF) are unable to inhibit activity of this truncated form of AR.

In control experiments, CPIC was able to down regulate the transcriptional activity of full-length AR transfected into COS cells (Fig 5.4A) or HeLa cells (Fig 5.4B), but not that of full-length glucocorticoid receptor (GR). This inhibition is not due to non-specific toxicity. In the same cell line, HeLa cells transfected with GAL-VP16 and treated with 10 μM CPIC show no

inhibition (Fig. 5.2). CPIC can directly target the N-terminal region in full-length or truncated forms of AR and potently inhibit AR variants often seen in aggressive prostate cancers.

***CPIC inhibits androgen-dependent and androgen-independent proliferation of CWR22Rv1 cells***

CWR22Rv1 is a human prostate cancer cell line derived from a transplanted xenograft that relapsed after castration-induced androgen ablation (21). CWR22Rv1 cells express both the full-length AR and two AR isoforms (10, 11). One of these variants, AR-V7 consists of the AR NTD, DBD and a C-terminal extension region (cryptic exons) (Figure 5.5A). AR-V7 is constitutively active and drives androgen-independent growth of the 22Rv1 cells and therefore, could play a role in castration resistance (9-12, 21). CPIC but not OHF inhibited androgen-independent proliferation of 22Rv1 cells, while both CPIC and OHF were able to reduce the androgen-dependent proliferation of 22Rv1 cells in 1 nM DHT (Fig 5.5B). Inhibition was similar to that reported for EPI-001, a compound derived from marine sponge which is also capable of interacting with the AR-NTD (Fig. 4D in (17)). CPIC represents a class of novel AR inhibitors that inhibit the proliferation of 22Rv1 cells, a model for CRPC, in the presence or absence of androgens by targeting the AR NTD rather than the LBD.

**DISCUSSION**

Constitutively active AR variants have been identified in aggressive prostate cancers and are associated with early recurrence and poor prognosis (22). These AR variants lack the hormone binding domain but are transcriptionally active in the absence of androgens, and elicit castration-recurrent growth of prostatic tumors. Using a cell-based high-throughput screen we identified the small molecule AR inhibitor, CPIC. Initially CPIC was thought to be a competitive antagonist of AR, much like bicalutamide and hydroxyflutamide. In radioligand binding assays, CPIC competed with 5 nM [$^3$H]-R1881 for binding AR at least as well as bicalutamide. Further analysis revealed that CPIC inhibits the AR N/C interaction much more potently than bicalutamide. The N/C interaction of AR plays a major role in the stabilization of the ligand-receptor complex as well as in AR dimerization and reduces receptor degradation (23). This raises the possibility that CPIC allosterically binds the AR NTD resulting in reduced N/C interaction and subsequently opposing androgen binding to the LBD.

We directly test this theory by analyzing the effects of CPIC on transactivation mediated by a constitutively active AR variant lacking the LBD. CPIC potently inhibits activities of AR that involve the N-terminal region. Inhibition of the constitutively active N-terminal AR was also seen using the less potent structurally related PIC19.7. In contrast, existing drugs Casodex (Bicalutamide) and Eulexin (hydroxyflutamide) are completely ineffective against this form of AR due to the absence of the LBD. Thus CPIC represents a family of compounds capable of directly targeting the N-terminal region in full-length or truncated forms of AR and potently inhibit AR variants often seen in CRPCs.

Another compound reported to target the N-terminal region of AR is EPI-001, identified from marine sponge by a group of scientists at the University of British Columbia, Vancouver (17). CPIC is at least 20 times more potent than EPI-001 in its ability to inhibit transactivation of N-terminal AR. Although through personal communications with Dr. Marianne Sadar, it is known that EPI-001 has gone through a rigorous process of lead optimization and a more potent analogue has been synthesized.

CPIC represents a potentially new class of therapeutics for castration recurrent prostate cancer. CPIC is active against the highly active truncated form of androgen receptor found in a significant percentage of the most aggressive and lethal prostate cancers. CRPC may contain primarily full-length AR, a mixture of full-length and truncated AR or exclusively truncated AR lacking the ligand-binding domain. CPIC is also very effective against full-length AR and inhibits the proliferation of AR positive cells. Because there are few therapeutic options for CRPC, interest in small molecule AR inhibitors with therapeutic potential is intense.

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**FIGURES**

**Figure 5.1**

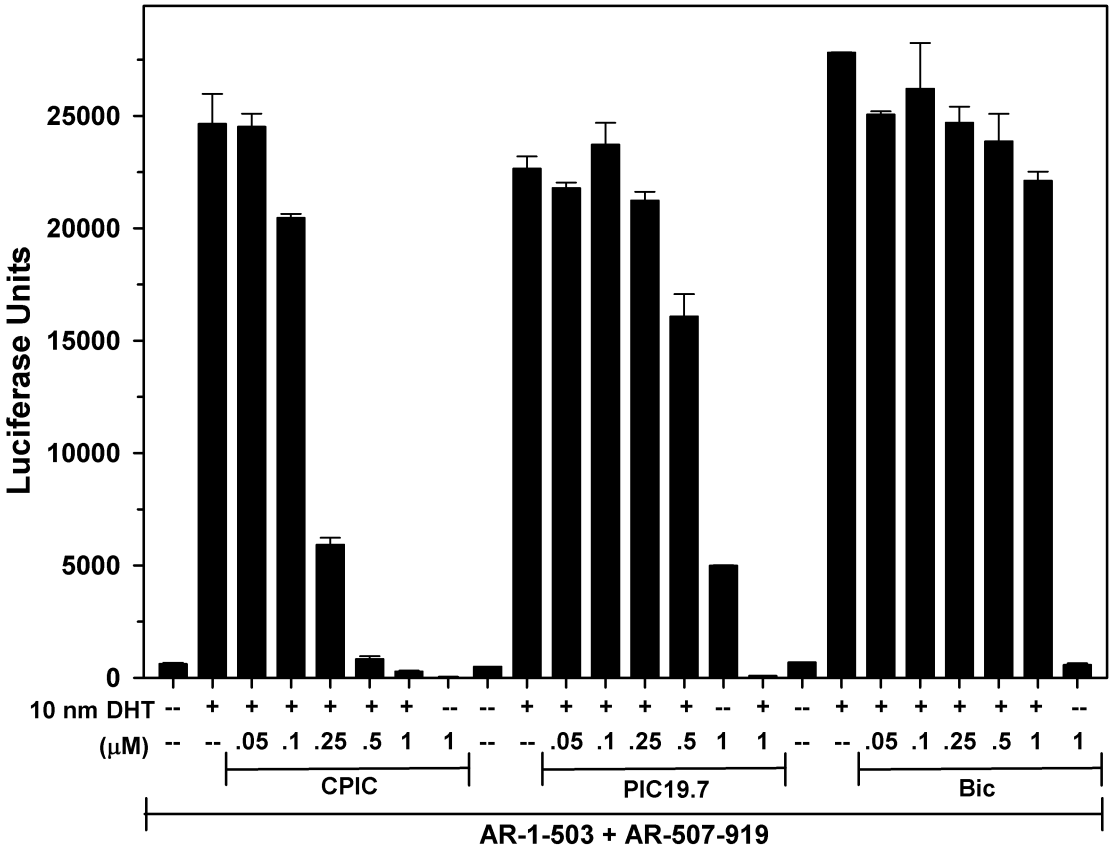


FIGURE 5.1. **CPIC inhibits transactivation by 2 fragments of AR linked by N/C interaction.** 50,000 HeLa cells/well were plated in 12-well plates. The cells were maintained in CD-FBS and transiently transfected with 50 ng of AR-507-919 and 50 ng of AR-1-503, and 100 ng of the androgen inducible PSA Enhancer-luciferase reporter. Transfected cells were maintained in the absence (-) or presence (+) of 10 nM DHT and the indicated concentrations (0-1 μM) of CPIC, PIC19.7 or bicalutamide/Casodex (Bic) and assayed for luciferase activity (Luciferase Units). Data is the average of 2 experiments performed in duplicates ± error. (Parts of this experiment are shown in Chapter 4, Figure 4.3C)

**Figure 5.2**

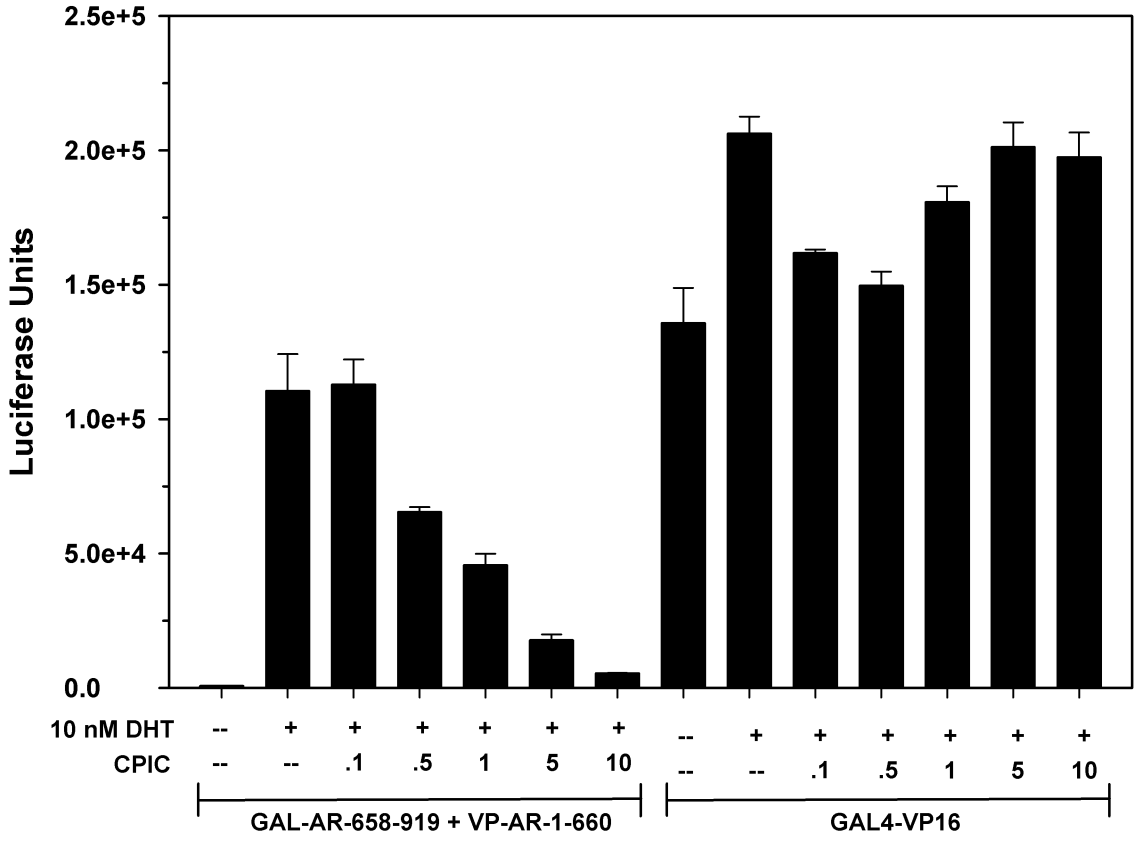


FIGURE 5.2. **CPIC inhibits the AR N/C interaction in 2-hybrid assays.** Mammalian 2-hybrid assays were carried out in transiently transfected HeLa cells. 50,000 HeLa cells/well were seeded in 6-well plates, maintained in CD-FBS and subjected to transient transfection using 50 ng/well of GAL-AR-658-919, 50 ng of VP-AR-1-660 and 100 ng of (GAL4)5-luciferase reporter, or as a control for non-specific effects of CPIC 50 ng of GAL-VP16 and 100 ng of (GAL4)5-luciferase. Transfected cells were maintained in the absence (--) or presence (+) of 10 nM DHT and the indicated concentrations of CPIC (0-10 μm) and assayed for luciferase activity (luciferase units). Data is the average of 2 experiments performed in duplicates ± Error.

**Figure 5.3**

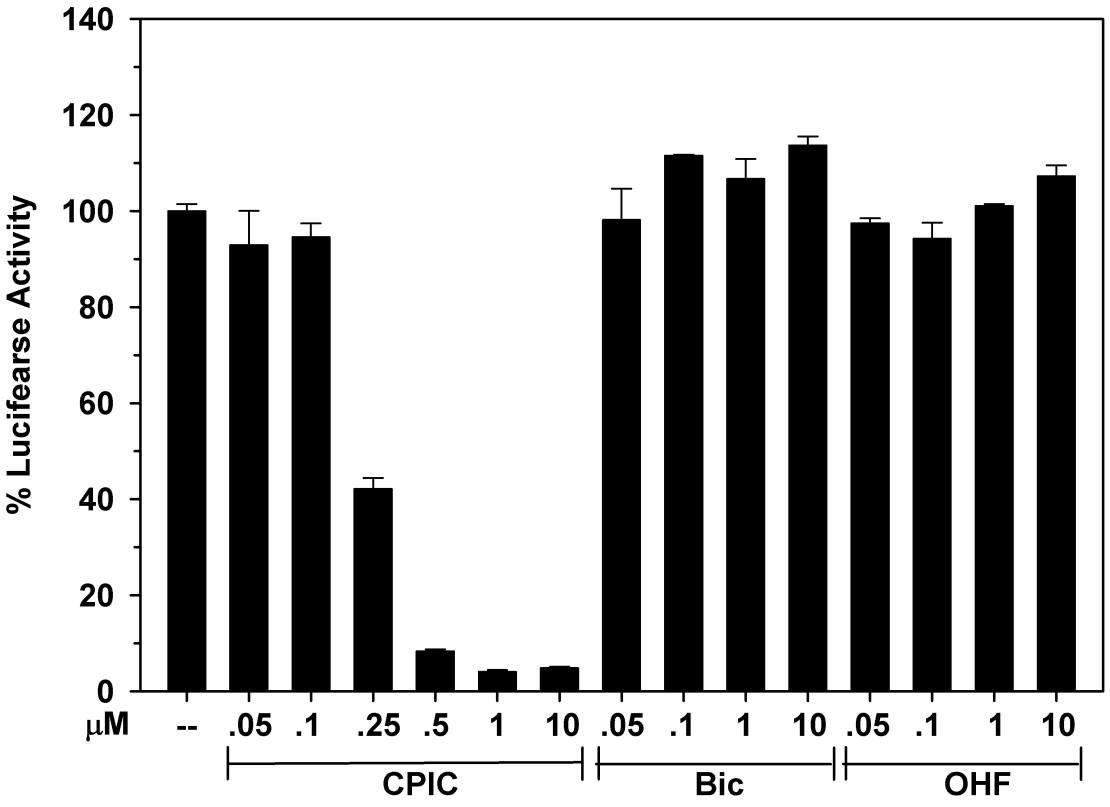


FIGURE 5.3. **CPIC potently inhibits the constitutively active N-terminal AR.** To compare the ability of CPIC, bicalutamide (Bic) and hydroxyflutamide (OHF) to inhibit transcription by the N-terminal AR, 50,000 HeLa cells/well were plated in 12-well plates and maintained in CD-FBS. The cells were transfected with 50 ng/well of AR-1-660 (contains the A, B and C domains of AR; the N-terminal region and the DNA-binding domain but lacks the ligand-binding domain) and 100 ng of the PSA Enhancer-luciferase reporter. Transfected cells were maintained with no added DHT and the indicated concentrations (0-10 μM) of CPIC, bicalutamide (Bic), or hydroxyflutamide (OHF) and assayed for luciferase activity (Luciferase Units). Data is the average of 2 duplicate experiments ± error.

**Figure 5.4**

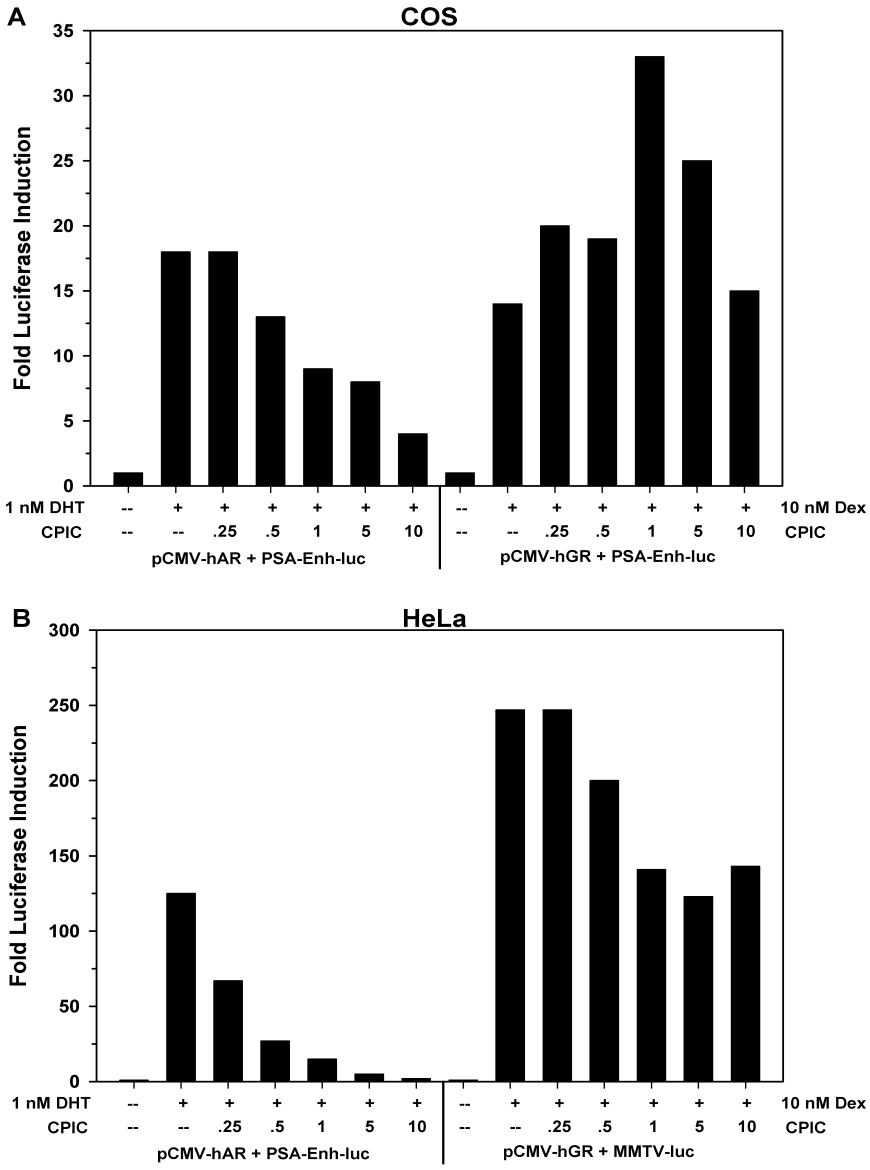


FIGURE 5.4. **CPIC specifically inhibits AR but not GR mediated luciferase activity.** A. 50,000 COS cells/well were plated in 12-well plates and maintained in CD-FBS. The cells were transfected with 10 ng/well of pCMV-hAR or pCMV-hGR along with 250 ng of the PSA Enhancer-luciferase reporter. B. 50,000 HeLa cells/well were plated in 12-well plates and maintained in CD-FBS. The cells were transfected with 10 ng/well of pCMV-hAR along with 250 ng of the PSA Enhancer-luciferase reporter or 10 ng/well pCMV-hGR and 100 ng of the MMTV-luciferase reporter. Transfected cells were maintained in the absence (--) or presence (+) of 1 nM DHT for AR or 10 nM Dex for GR and the indicated concentrations (0-10 μM) of CPIC and assayed for luciferase activity (Luciferase Units). Data is the average fold induction of 2 duplicate experiments.

**Figure 5.5**

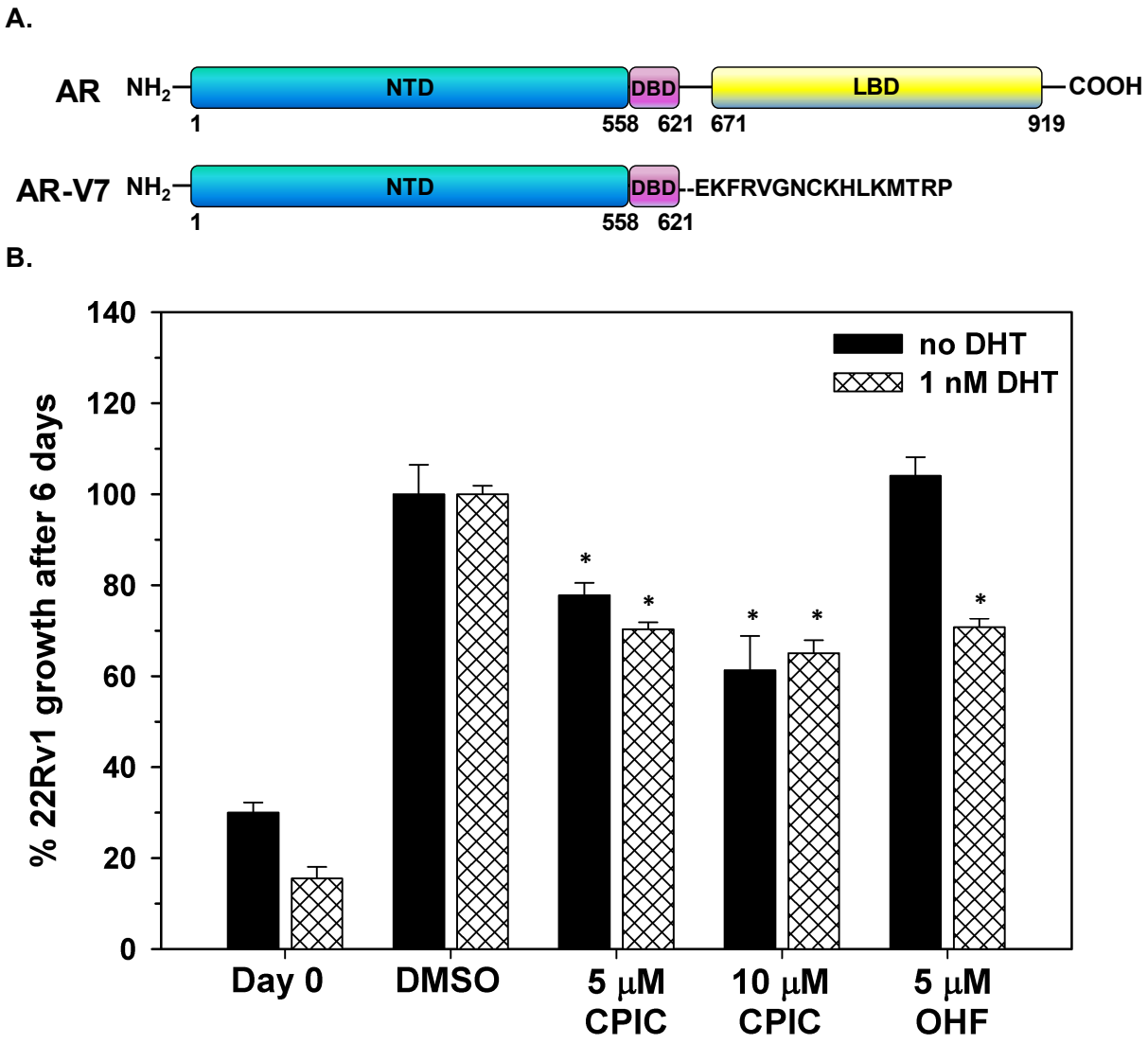


FIGURE 5.5. **CPIC inhibits androgen-dependent and androgen-independent proliferation of 22Rv1 cells.**

A. Schematic diagram of the domains of full-length AR and the splice variant AR-V7 expressed in 22Rv1 cells. B. 22Rv1 cells were stripped for 4 days in 5% CD-FBS containing media and plated at 2000 cells/well in 96-well plates. Cells were maintained without added androgens (no DHT) or with 1 nM DHT and the indicated concentrations of CPIC or OHF and assayed using MTS after 6 days. Cell growth in DMSO after 6 days was set to 100% and data points represent the mean of at least 3 wells ± SEM. Significance was tested using Student's T-test and $p < 0.05$.

# CHAPTER 6
# A SMALL-MOLECULE INHIBITOR THAT REDUCES THE LEVEL OF ANDROGEN RECEPTOR

Androgen receptor (AR) remains a key target in castration-recurrent prostate cancer (CRPC). The molecular mechanisms driving the reactivation of AR in this malignant state are not fully understood. Some potential causes for the CRPC phenotype includes the expression of AR splice variants or gain of function mutants of AR, which are constitutively active in the absence of ligands. This underscores the need to identify new molecules not only able to bind to the full-length AR, but also capable of inhibiting transactivation by AR splice-variants and mutants.

We performed a stringent high-throughput screen using an androgen-induced luciferase-reporter system in a stable HeLa cell line, which allowed us to identify AR54 (2-(pyrimidin-2-ylthio)-1-(2,2,4-trimethyl-4-phenyl-3,4-dihydroquinolin-1(2H)-yl)ethanone) as a novel non-competitive inhibitor of AR action. In LAPC-4 prostate cancer cells, AR54 blocks cell proliferation, reduces the level of AR mRNA and subsequently down-regulates AR protein levels. We have not yet determined whether AR54 reduces the production of AR transcripts or increases the rate of mRNA degradation. AR54 is able to inhibit both androgen-dependent and androgen-independent proliferation of CWR22Rv1 cells, presumably by inhibiting the transactivation potential of both full-length AR and constitutively active AR variants present in these cells.

## INTRODUCTION

Androgens, acting through the androgen receptor (AR), play a key role in the development and progression of prostate cancer (PCa). Antiandrogen and androgen deprivation therapy have been standard therapies for metastatic and recurrent prostate cancer since Huggins and Hodges (1) reported the effect of castration on PCa. Although most patients initially respond favorably, over time tumors recur as aggressive castration-resistant or castration-recurrent prostate cancer (CRPC) with limited therapeutic options in approximately 25% of patients (2). The mechanisms underlying castration-resistant androgen receptor (AR)–mediated signaling remain unclear, although several possible mechanisms have been proposed including AR gene mutation,

increased AR expression, or increased androgen biosynthesis in prostate tumors (3-8). In spite of intensive androgen-depleting therapies used to mitigate the cancer at the initial stages of progression, continued AR signaling is a common feature of CRPC and AR remains a key therapeutic target (2, 3). A thorough understanding of the how AR activity promotes tumor growth and survival is the need of the hour.

As a transcription factor, the AR regulates the expression of many coding and noncoding RNA targets. In CRPC, transactivation by AR can occur through a number of interdependent mechanisms, including increased expression of the receptor, generation of mutations in the receptor, and altered action of coregulatory proteins, through cross talk with other signaling pathways or through the action of constitutively active splice variants (6). Current antiandrogens used in the clinic, including bicalutamide (Casodex) and flutamide (Eulexin), specifically act by competing with androgens for binding the AR. Blockage of androgen binding results in inhibition of AR-mediated gene expression and cell proliferation, but transactivation of the receptor by other factors renders these treatments ineffective in advanced PCa. In model settings of CRPC bicalutamide undergoes an antagonist-to-agonist switch, stimulating AR activity and prostate tumor cell growth (9). There is also some evidence to demonstrate that AR mutations are selected in response to strong selective pressure from flutamide treatment (10). The continued viability of AR as a therapeutic target is illustrated by the recent approval of the inhibitor of androgen biosynthesis, abiraterone, which prolongs survival in late-stage metastatic prostate cancer (11), MDV3100 which acts by competing with androgens for binding in the ligand-binding pocket of AR and by the ongoing efforts to develop ARN-509, also a competitive antagonist with a better dose-response profile in mouse xenograft tumors (9, 12). Most efforts to target AR action focus on inhibition of androgen synthesis, or on improved antiandrogens. Efforts have also been intensified in finding natural chemicals that can act as ligand inhibitors of therapeutic targets like AR. C Chang and colleagues describe such an AR inhibitor, ASC-J9, which is a curcumin derivative capable of enhancing AR degradation (13). In our hands, ASC-J9 was a potent inhibitor of AR action but it also exerted substantial indiscriminate toxic effects in AR-negative environments in the same concentration range (data not shown).

Because there are several mechanisms by which AR retains its ability to act in CRPC (14), we were interested in identifying noncompetitive small-molecule inhibitors that reduce AR levels. After screening a library of about ~160,000 small molecules, we identified a potent

noncompetitive inhibitor of AR in prostate cancer cells. This compound, AR54 (2,2,4-trimethyl-4-phenyl-1-[(2-pyrimidinylthio)acetyl]-1,2,3,4-tetrahydroquinoline), was able to down-regulate AR protein levels in prostate cells. A dozen small molecules that were structurally related to AR54 were also tested for potency, specificity and toxicity. By reducing AR levels, our non-competitive small molecule inhibitor AR54 should be able to target most known mechanisms by which AR can act in CRPC.

**EXPERIMENTAL PROCEDURES:**

*Chemical Libraries*- The libraries screened were the ChemBridge MicroFormat small molecule library obtained from ChemBridge™ containing ~150,000 small molecules, the Marvel library, developed at the University of Illinois by K. Putt and P. Hergenrother containing ~9,700 small molecules (15, 16) and the NCI Diversity Set from NIH containing ~1,990 small molecules.

*Cell Culture*- AR-positive cell lines included LNCaP and LAPC-4 human prostate cancer cells maintained in phenol-red free RPMI 1640 with 10% fetal bovine serum (FBS; Atlanta Biological, GA). LAPC-4 growth medium were routinely supplemented with 1 nM of the synthetic androgen methyltrienolone (R1881). Cells were transferred to RPMI 1640 containing 5% charcoal-dextran stripped (CD) FBS at least three days prior to plating for an experiment. CWR-R1 cells (17) were grown in modified iMEM (GIBCO#10488-001) containing 2.5 g/l of glucose, 1.2 g/l Niacinamide, 0.5 ml of Insulin-Transferrin-Selenium (ITS; Roche #11074547001), 10 ng/mL epidermal growth factor (EGF) and 2% FBS. The cells were transferred to medium containing 2% CD-FBS without EGF 3-4 days before the experiment. CWR22Rv1 cells were obtained from ATCC and maintained in RPMI 1640 supplemented with 10% FBS for growth flasks or 5% CD-FBS for the treatment flasks and experimental plates.

AR-negative cell lines included PC3 human prostate cancer cells maintained in RPMI 1640 with 10% FBS, and DU145 human prostate cancer cells and MDA-MB-231 human breast cancer cells grown in MEM with 10% FBS.

Other cell lines included estrogen receptor α (ERα)-containing T47D-KBluc breast cancer cells (18) expressing an $(ERE)_3$-luciferase reporter gene , maintained in RPMI 1640 containing 10% FBS. Three days before induction with 17β-estradiol ($E_2$), cells were transferred to medium, containing 5% CD-FBS. T47D/(A1-2) cells stably express the human glucocorticoid receptor

(GR) and contain a mouse mammary tumor virus (MMTV)-luciferase reporter (19) were maintained in MEM, 5% FBS and 0.2 mg/ml G418. The MMTV-Luc reporter is inducible by AR, progesterone receptor (PR) and GR depending on the activating ligand used. Before the experiment, cells were transferred to the above medium containing 5% CD-FBS.

The RPMI Base medium was supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM Hepes, and pH 7.5, 1 mM sodium pyruvate. MEM was supplemented with 10 mM HEPES, pH 7.4 and 2 mM glutamine.

All cells were maintained at 37˚C in 5% $CO_2$ in growth medium containing 1% penicillin and streptomycin and 2-10% fetal bovine serum (FBS) (Atlanta Biological, Atlanta, GA) without phenol-red.

*Reporter Gene Assays*- At least 3 days before each experiment cells were transferred to medium containing CD-FBS as described above. T47D/(A1-2) and T47D-KBluc cells (200,000 cells/well) were plated in 1 ml of medium in 24-well plates. After 24 h the indicated concentrations of $E_2$, or dexamethasone (Dex) were added along with each inhibitor or DMSO (vehicle). After 24 h, cells were lysed in 100 µl of Passive Lysis Buffer (Promega, Madison WI) and luciferase activity was determined using BrightGlo firefly luciferase reagent (Promega). Unless otherwise mentioned, total DMSO (vehicle) concentration in all assays was maintained at or below 0.1%.

*Cell Growth and Viability Assays*- To assay cell growth, 2,000 cells/well were plated in 96 well plates. LNCaP and LAPC-4 cells were maintained in CD-treated serum for at least 2-4 days prior to each experiment. All AR negative cell lines, MDA-MB-231 and PC3, were plated in growth medium 24 hours prior to treatment. Treatment medium containing vehicle or the indicated concentrations of R1881, with or without inhibitor compounds was added to the cells and incubated for the indicated number of days. Cell viability was determined using Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS).

*Radioligand Binding Assay*- Competitive radioligand binding assays were performed by expressing pCMV-AR in monkey kidney COS cells and incubating cell cultures for 2 h at 37˚C with [$^3$H]R1881 (17α-methyl-[$^3$H] methyltrienolone, 82 Ci/mmol) in the absence and presence of competitor ligands (20).

*Endogenous Gene Expression*- LNCaP, LAPC-4 and MCF-7 cells were seeded into 6-well plates and grown for 3-4 days in medium containing 5% CD-FBS. Cells were then treated with

ethanol or hormone, along with the indicated concentrations of the inhibitor for 24 hours. RNA was extracted and purified using the Qiagen RNeasy kit. cDNA was prepared from 1 μg of RNA with M-MuLV reverse transcriptase from NEB. Diluted cDNA was used to perform quantitative RT-PCR using SybrGreen (ABI Thermocycler) with actin as the internal standard. Primers for qRT-PCR were: β-actin forward primer 5'- TGT CAC CAA CTG GGA CGA CA and reverse primer 5'- GGG GTG TTG AAG GTC TCA AA; PSA (kallikrein 3) forward primer 5'- GGT GAC CAA GTT CAT GCT GTG and reverse primer 5'- GTG TCC TTG ATC CAC TTC CG; TMPRSS2 forward primer 5'- TAG TGA AAC CAG TGT GTC TGC and reverse primer 5'- AGC GTT CAG CAC TTC TGA GGT CTT; PS2 forward primer 5'- ACC GGA CAC CTC AGA CAC G 3' and reverse primer 5'- CTG TGT TGT GAG CCG AGG C 3'and ERα forward primer 5'- GCG AAG AGG GTG CCA GGC TTT 3' and reverse primer 5'- ATG GAG CGC CAG ACG AGA CCA 3'.

*Western Blot*- Cells were plated at 300,000 cells/well in 6-well plates in medium containing 5% CD-FBS. The medium was changed on day 2, and on day 4 the cells received fresh medium containing the indicated treatments. Whole cell extracts were prepared after 24 h of treatment using 1X RIPA buffer (Millipore, CA) containing complete mini protease inhibitor cocktail (Roche, Germany). 30 μg of protein per lane was analyzed on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (GE Healthcare). AR protein was detected using AR antibody AR (441) (sc-7305, Santa Cruz, CA) and β-actin was detected using monoclonal antibody A1978 (Sigma).

*Statistical Analysis*- Results are expressed as mean ± SEM of at least three independent experiments. Significance was established when $p<0.05$. Student's t test was used for comparison of the means between two groups.

## RESULTS

***AR54 is a Novel non-ligand Small-molecule Inhibitor of AR in Prostate Cancer Cells-*** To identify small molecules that inhibit the growth of AR positive cancer cells we carried out high-throughput screening (HTS) of a ~160,000 small molecule library at the UIUC HTS Center. Validated small molecule "hits" were evaluated for potency, efficacy, specificity and toxicity. AR54 (Fig 6.1A) is one of the most effective novel small-molecule inhibitor to emerge from this study. The ability of AR54 to compete with R1881 binding to AR was evaluated in COS cells

transfected with wt-hAR. In competitive radiometric binding assays AR54 did not compete with the synthetic radiolabeled androgen R1881 for binding at the AR ligand-binding domain (Fig 6.1B). Therefore AR54 most probably acts outside of the ligand-binding pocket of AR. Interestingly, in analyzing a cohort of 13 compounds sharing >85% structural similarity with AR54(Supplemental Table 6S.1), we found that one compound ARD67 competes with androgens for binding AR and shows the phenotype of an AR inhibitor (Supplemental Fig 6S.1). Comparisons of the activities of these analogues on AR dependent and independent proliferation of various cell lines are summarized in Supplemental Fig 6S.2 and may serve as a limited structure-activity correlation study.

***AR54 Inhibits Growth of AR Positive LAPC-4 Prostate Cancer Cells and Exhibits Low Toxicity in AR Negative Cells-*** We evaluated the effect of AR54 on the androgen-dependent proliferation of some AR-positive cell lines. LAPC-4 cells contain wild-type AR. In dose response studies, AR54 inhibited androgen-dependent growth of LAPC-4 cells with an $IC_{50}$ of 3.5 μM (Fig 6.2A). In 7-day experiments with 10 μM and 20 μM AR54 in the absence of R1881, the final number of cells were similar to that seen in LAPC-4 cells grown with vehicle DMSO without R1881. This indicates indirectly that AR54 does not elicit an indiscriminate toxic effect in cells.

Fig 6.2B compares AR54 with other known inhibitors of AR and shows that in these cells AR54 was more effective than the competitive AR inhibitor ASC-J9, which also causes AR degradation (13).

In PC3 cells, AR54 did not inhibit cell growth at 10 μM and did not reach 50% inhibition at the highest concentration tested, 30 μM (Fig 6.2C). We showed that MDA-MB-231 cells are highly susceptible to non-specific damage (15) and AR54 was less toxic than the competitive AR inhibitor ASC-J9 (Fig 6.2D). The results indicate that at concentrations of AR54 sufficient for inhibition of AR-mediated proliferation of AR-positive prostate cancer cells, it exerts minimal toxicity on AR-negative cells.

***AR54 specifically inhibits AR action-*** AR sequence has substantial structural homology with other steroid receptors namely the GR and PR. Therefore, from a therapeutic and mechanistic point of view, it is important for an inhibitor to exhibit specificity in binding AR without affecting the ubiquitously expressed GR.

We evaluated the effect of AR54 on transcription mediated by other steroid receptors in cell lines stably transfected to express reporter genes. Inhibition of ERα, was evaluated in T47D-KBluc cells that express endogenous ERα and are stably transfected with an estrogen ($E_2$) inducible $(ERE)_3$-luciferase reporter gene. Inhibition of glucocortioid receptor (GR) was evaluated in T47D/(A1-2) cells that are stably transfected to express GR and a mouse mammary tumor virus (MMTV)-luciferase reporter gene. In these cells treatment with the synthetic glucocorticoid, dexamethsone (Dex) activates the MMTV reporter through GR. 10 μM AR54 treatment results in minimal inhibition of ERα and GR mediated transactivation but 20 μM AR54 reduces ERα mediated luciferase activity (Fig 6.3A).

To ensure AR54 specificity for AR inhibition at concentrations ≤10 μM, it was also probed in MCF-7 cells that contain abundant ERα, transactivation by which is inducible by $E_2$. Treatment with 10 μM AR54 for 24 hours did not alter the expression of the ERα-regulated PS2 gene transcripts (Fig 6.3B).

At concentrations sufficient for inhibition of AR action, AR54 had little effect on two other members of the family of ligand activated nuclear receptors, GR or ERα. The results suggest that AR54 is a relatively specific inhibitor of AR-mediated transcription at or below 10 μM.

***AR54 inhibits Expression of Endogenous Androgen-regulated Genes -*** To evaluate the effect of AR54 on endogenous gene expression, the levels of mRNAs for several well–characterized androgen-related genes were measured in LNCaP and LAPC-4 cells. In LNCaP cells, the *PSA* and *TMPRSS2* genes are highly induced and *RDC1* gene transcripts are down regulated by androgens working through AR. In LNCaP cells treated for 24 h, 10 μM AR54 strongly inhibited the induction of PSA and TMPRSS2 mRNAs (Fig 6.4A) while reversing the androgen-induced reduction of RDC1 mRNA (Fig 6.4B). We also note that in the absence of androgens, AR54 treatment does not result in AR transactivation in LNCaP cells, thus indicating that it is a pure antagonist of AR action.

AR54 also reduced the expression of PSA mRNA in a dose-dependent manner with an $IC_{50}$ of ~3 μM in LAPC-4 cells (Fig 6.4C). In summary, AR54 is able to inhibit AR-dependent proliferation and gene expression in LAPC-4 cells with a half maximal concentration of ~3 μM.

***AR54 Down-regulates AR Levels***- Hypothetically, reduction of all AR signaling in tissue can be achieved by treatment with a small molecule that specifically and potently increases AR degradation. We analyzed AR protein levels from LAPC-4 cells treated with AR54. Western blot

analysis shows that in the presence and absence of androgen, 10 μM AR54 (Fig 6.5A, B, C) down-regulates AR protein levels. AR54 is at least as effective as ASC-J9, a curcumin derivative shown to increase AR degradation (13). Other AR inhibitors we identified, AR45 and AR52 did not down-regulate AR and serve as controls (Fig 6.5A). A time-course performed with 10 μM AR54 also shows reduced amounts of total AR when compared to R1881 treated control wells as early as 4 h after treatment (Fig 6.5C).

Further analysis revealed that AR54 reduces AR mRNA levels in LAPC-4 cells (Fig 6.5D). This down regulation of AR mRNA is presumably responsible for the reduction in AR protein levels and seems to be specific for AR when compared to ERα. AR54 is unable to alter the levels of ERα mRNA in MCF-7 cells treated with estradiol (Fig 6.5E). We have tried to use actinomycin-D in LAPC-4 cells to dissect whether AR54 causes an increased degradation of AR mRNA, but have been unsuccessful in these experiments so far possibly due to the cell-damage elicited by actinomycin-D.

***AR54 Modestly Reduces Androgen-Independent Proliferation of CWR22Rv1 Cells-*** CWR22Rv1 is a CRPC cell line derived from the CWR22R subline, which was isolated from the recurrent tumor of the androgen-dependent CWR22 cells in castrated mice (21). This cell line contains full-length AR along with LBD-truncated AR splice variants. Some of these AR splice variants have been shown to have transactivation capacity similar to the full-length AR and are capable of eliciting androgen-independent proliferation in 22Rv1 cells.

We also assessed the effects of treating 22Rv1 cells with 10 μM AR54. The results also showed that 1 nM DHT can promote cell growth modestly and AR54 suppressed the DHT-induced cell growth (Fig 6.6A). Although the inhibitory effect of AR54 on 22Rv1 cell proliferation is slight, we are encouraged by the fact that these are comparable to what other researchers have observed in these cells (22, 23).

We also examined the effects of AR54 on full-length AR and truncated AR-mediated targeted gene expression. 1 nM DHT modestly induced the expression of some AR-targeted genes in 22Rv1 cells. Addition of 10 μM AR54 suppressed PSA and KLK2 gene-expression in both DHT-mediated and androgen-independent environments.

## DISCUSSION

The AR remains a key target in CRPC. AR54, a novel compound, which acts by down-regulating AR gene transcripts, has potential to show effectiveness against all AR-mediated actions in CRPC. AR54 is relatively specific for down-regulation of AR. Due to the substantial structural homology of the AR sequence with other steroid receptors, it is important for an inhibitor to exhibit specificity in binding AR without affecting the ubiquitously expressed GR, from a therapeutic and mechanistic point of view. For example, the last decade saw renewed interest in the ansamycin antibiotic 17-allylamino, 17-demethoxygeldanamycin (17-AAG) which was discovered to be an anti-tumor agent that binds and inhibits Hsp90. 17-AAG is able to disrupt the Hsp90-protein complexes and lead to the degradation of the associated proteins. A growing number of Hsp90 client proteins have been shown to be important for the development, proliferation and survival of several types of cancer, including prostate cancer. Inhibition of Hsp90 leads to the degradation of known oncogene products, such as Her2, BRAF, Erk1/2 and others, leading to the simultaneous blockade of multiple oncogenic transduction pathways (24). But the nonspecific disruption by 17-AAG of the interaction between Hsp90 and most of its associated proteins leads to extensive adverse and unwanted side effects including anorexia, diarrhea, nausea, fatigue, vomiting and the reversible elevation of liver enzymes and therefore limits the applicability of 17-AAG as a therapeutic molecule.

If AR54 exhibited general toxicity in cells it might be expected to indiscriminately block R1881-bound AR induced target gene expression. The ability of AR54 to reverse both R1881-AR mediated up regulation of target mRNAs (PSA and TMPRSS2) and down regulation of RDC1 mRNA provides evidence that AR54 is acting as a specific inhibitor of AR and not through non-specific toxic mechanisms.

Importantly, by reducing AR mRNA levels, AR54 is likely to enhance effectiveness of current agents in resistant cells. Bicalutamide is a potent agonist in LNCaP/AR cells that overexpress AR, but is a full antagonist in the parental LNCaP cells expressing several fold less AR. By reducing AR levels, it is possible that AR54 could restore the antagonist activity of bicalutamide in some resistant cells.

It remains unclear whether AR54 directly binds AR at all. Some evidence points to the possibility of AR54 suppressing AR action by altering some crucial signaling pathway. The possibility that AR54 changes the activity of certain microRNAs, resulting in reduced AR

transcript levels cannot be ruled out either. We are encouraged to pursue further mechanistic studies of AR54 action by its specificity for AR compared to ERα and GR.

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**FIGURES:**

**Figure 6.1**

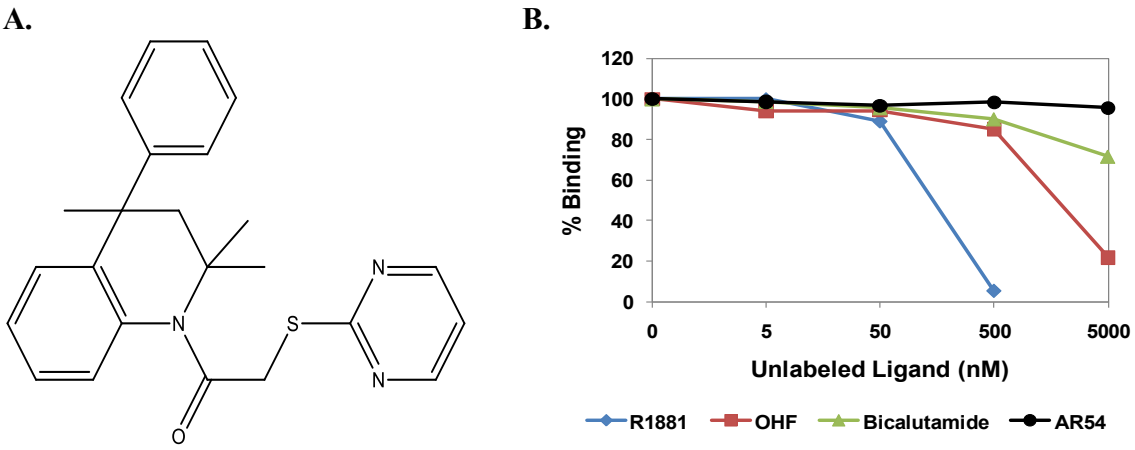



FIGURE 6.1. **AR54 does not compete with androgens for binding to AR.** *A*, Structure of AR54. *B,* Radioligand competition assay. Ligand-competition assays were performed using 5 nM [$^3$H]-R1881 and indicated concentrations of unlabeled small molecules. In contrast to R1881 and the antiandrogens hydroxyflutamide (OHF) and bicalutamide, AR54 did not compete with labeled R1881 for binding to AR.

**Figure 6.2**

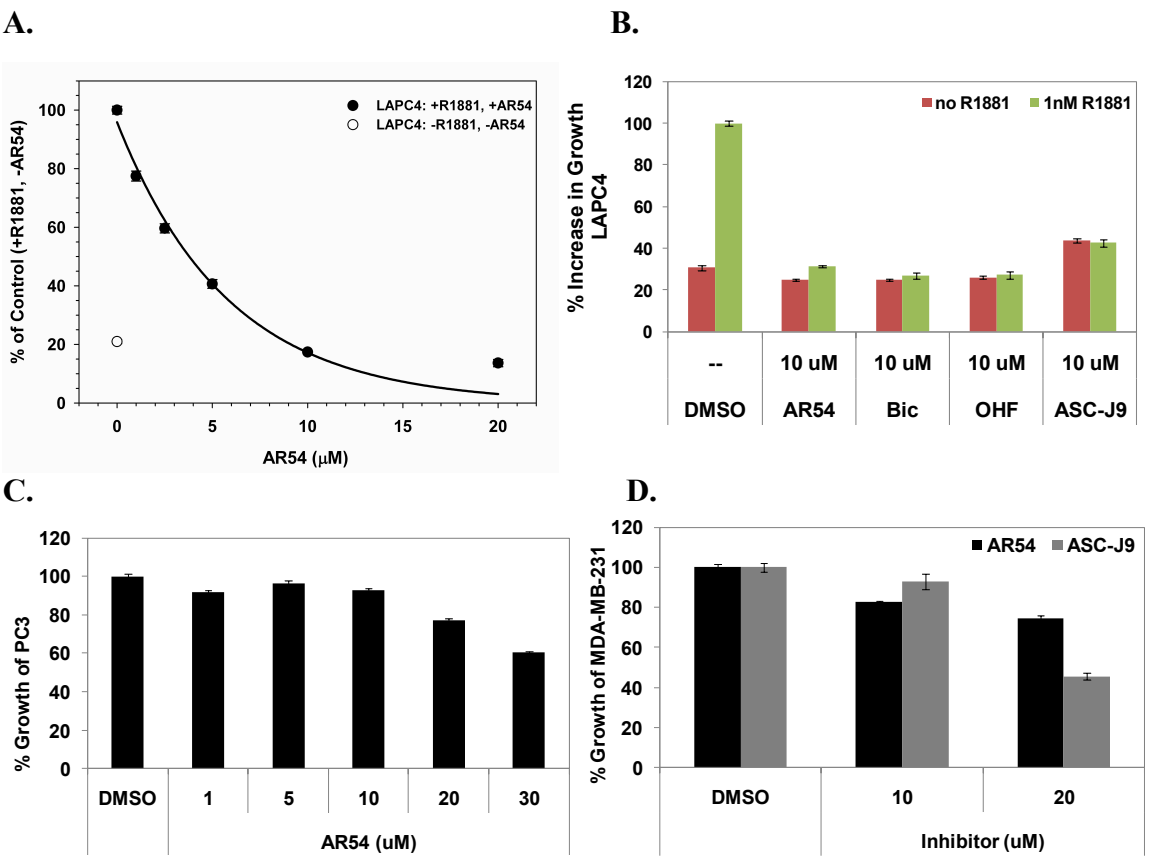



FIGURE 6.2. **Inhibition of R1881-dependent prostate cancer cell growth by AR54**. Dose-response study of AR54 inhibition of cell growth in LAPC-4 cells (*A* and *B*), PC3 cells (*C*), and MDA MB-231 cells (*D*). Adaptation to R1881-free medium, growth and calculation of cell numbers and MTS assays were as described in Chapter 3. LAPC-4 cells were maintained in medium containing 5% CD-FBS with (filled circles) or without (open circle, vehicle only) 1 nM R1881. PC3 and MDA-MB-231 cells were cultured in medium containing 10% FBS. Data is the average of 8 wells of cells ± S.E.M. The number of cells in 1 nM R1881+DMSO was set at 100%. The $IC_{50}$ for inhibition of LAPC-4 cell growth was calculated using Sigma Plot. (Data in Figure 6.2A: Khin Khin Soe H Wu)

**Figure 6.3**

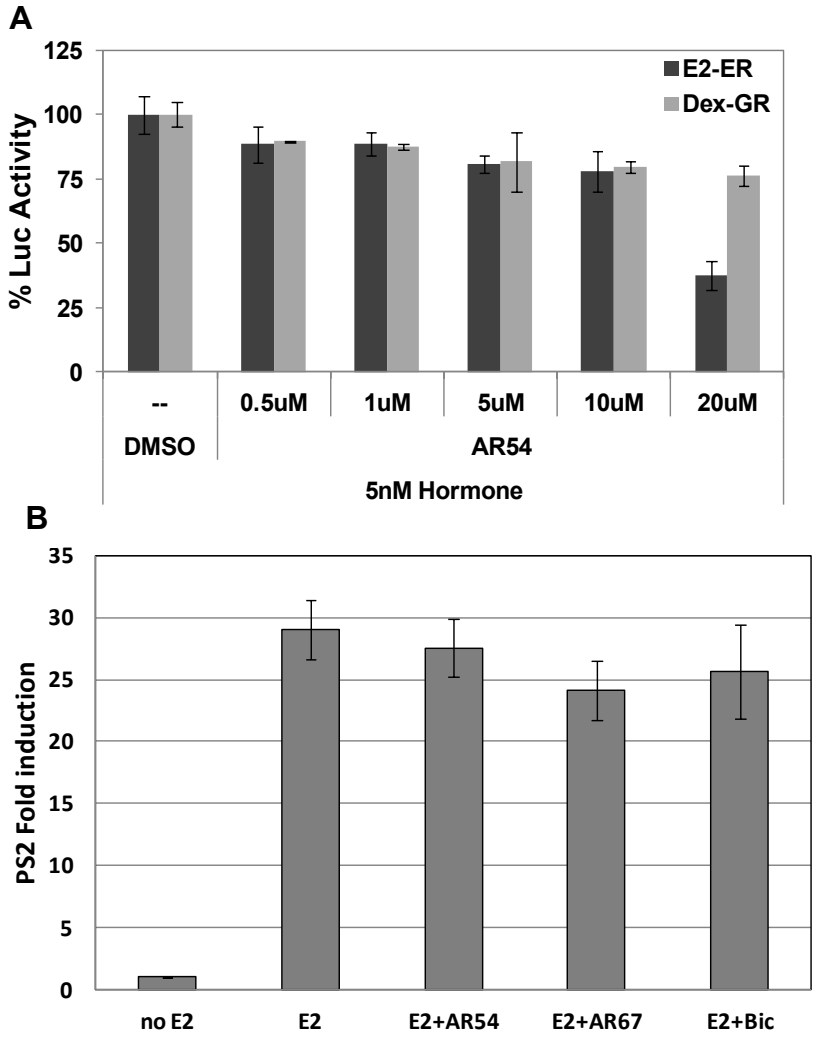



FIGURE 6.3. **AR54 does not inhibit other steroid receptors at 10 μM.**

*A*, Two different stably transfected T47D stable cell lines were used to gauge the effect of AR54 on ERα and GR mediated luciferase activity. Data is the mean of 3 independent experiments ± SEM. *B*, MCF-7 cells were plated in medium containing 5% CD-FBS, treated with 10 nM estradiol ($E_2$) and 10 μM of the indicated inhibitors for 24 h before RNA extraction. mRNA was quantitated using qRT-PCR and normalized to β-actin. Data represent the mean of 3 independent experiments ± SEM.

**Figure 6.4**

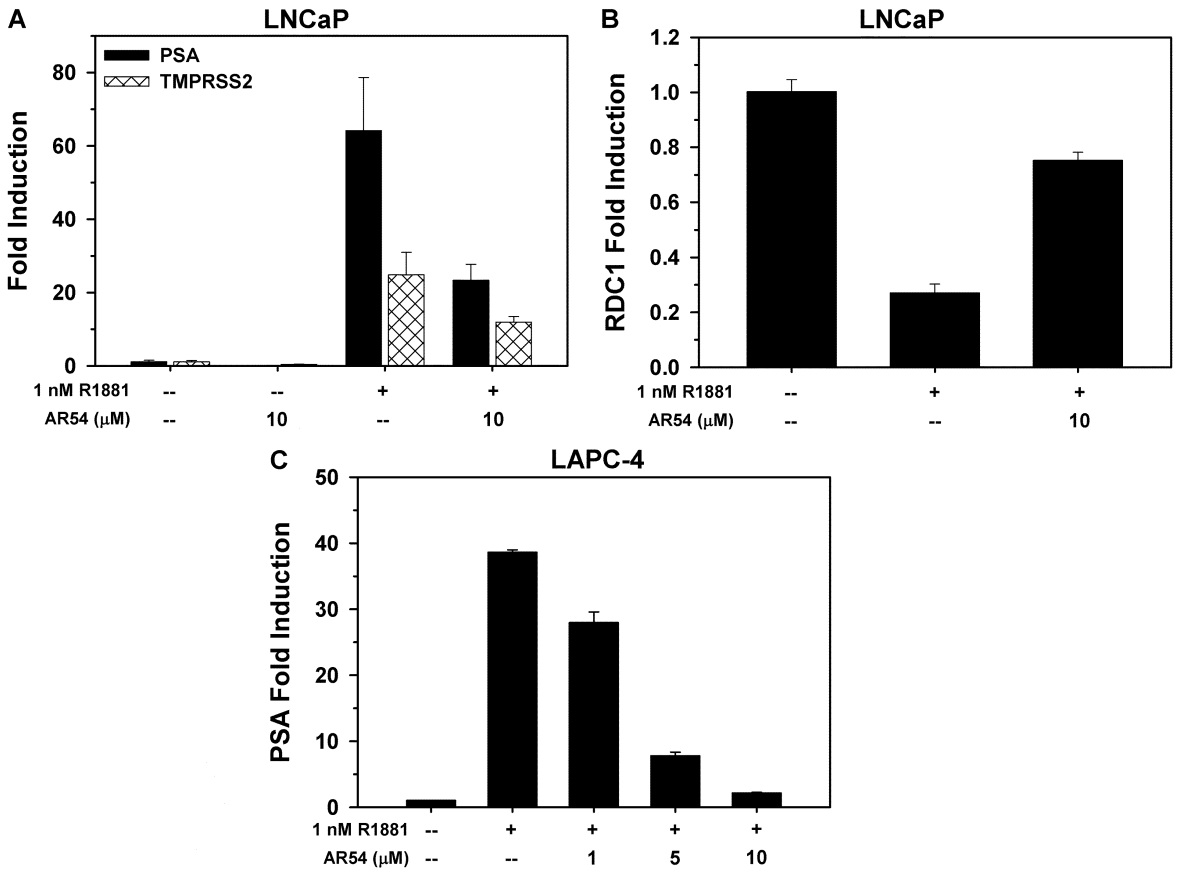



FIGURE 6.4. **AR54 inhibits induction of AR-regulated genes**. Hormone-depleted LNCaP (*A,B*) and LAPC-4 (*C*) cells were treated for 24 h and mRNA levels determined by qRT-PCR using β-actin to normalize. Data is the mean of 3 independent experiments ±SEM.

**Figure 6.5**

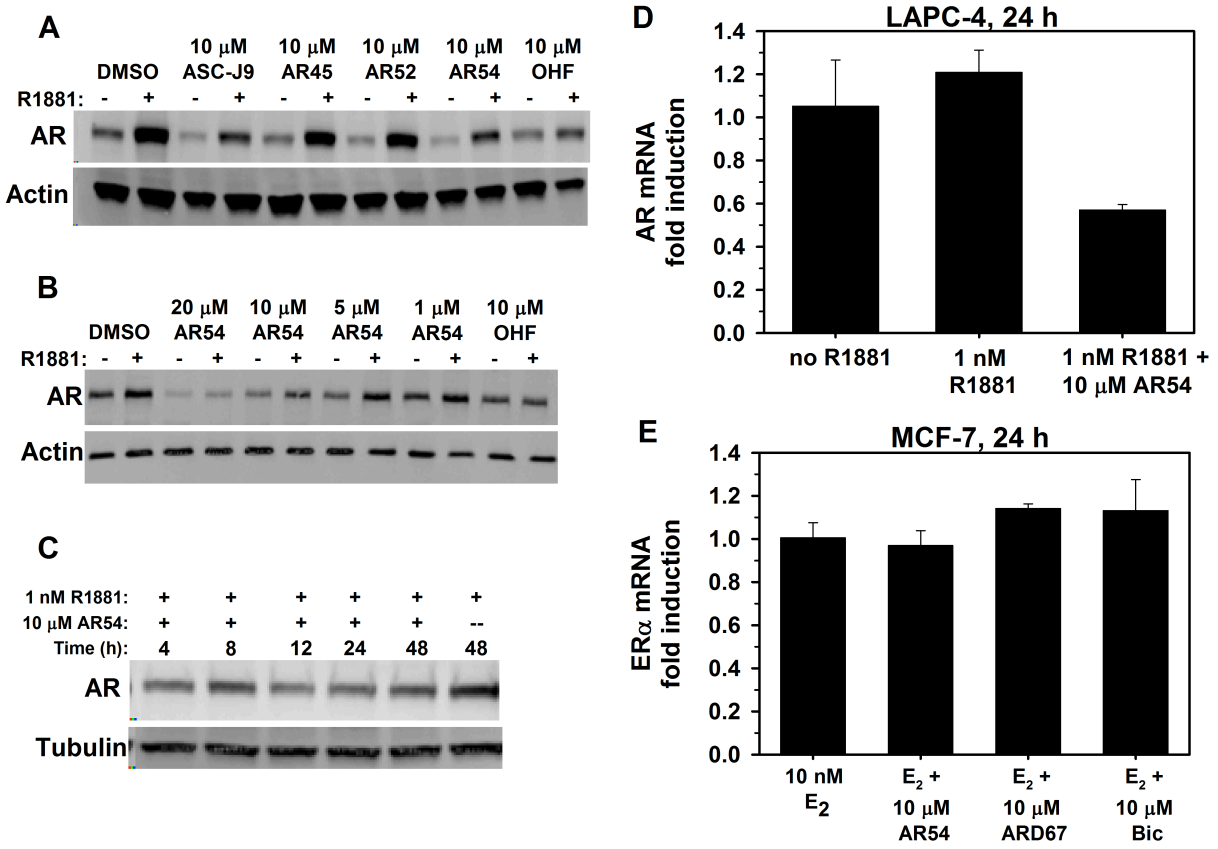



FIGURE 6.5. **AR54 down-regulates AR.** *A*, Hormone-depleted LAPC-4 cells were maintained with or without R1881 with DMSO or 10 μM inhibitor for 24 hours. 10% polyacrylamide gel wells were loaded with equal amounts of protein in each lane, fractionated and analyzed by western blotting. *B*, LAPC-4 cells were treated with 5, 10 or 20 μM AR54 or 10 μM hydroxyflutamide (OHF) in the absence (-) or presence (+) of 1 nM R1881. Equal amounts of protein was loaded on 10% polyacrylamide gels, fractionated and analyzed by western blotting. *C*, LAPC-4 cells were treated with 10 μM AR54 or DMSO (--) in the presence (+) of 1 nM R1881 for the indicated amount of time (4 h, 8 h, 12 h, 24 h, and 48 h). Cells were lysed and protein analyzed by western blotting. The antibody used to detect AR was AR441 monoclonal antibody (NeoMarkers, Freemont, CA). *D*, Hormone-depleted LAPC-4 cells were treated for 24 h and AR mRNA levels determined by qRT-PCR using β-actin to normalize. Data is the mean of 3 independent experiments ±SEM. E, MCF-7 cells were treated with 10 nM estradiol (E2) for 24

h in the presence of 10 μM of indicated AR inhibitors (AR54, ARD67 or bicalutamide (Bic)). ERα mRNA levels determined by qRT-PCR and normalized to β-actin. Data is the mean of 3 independent experiments ±SEM.

**Figure 6.6**

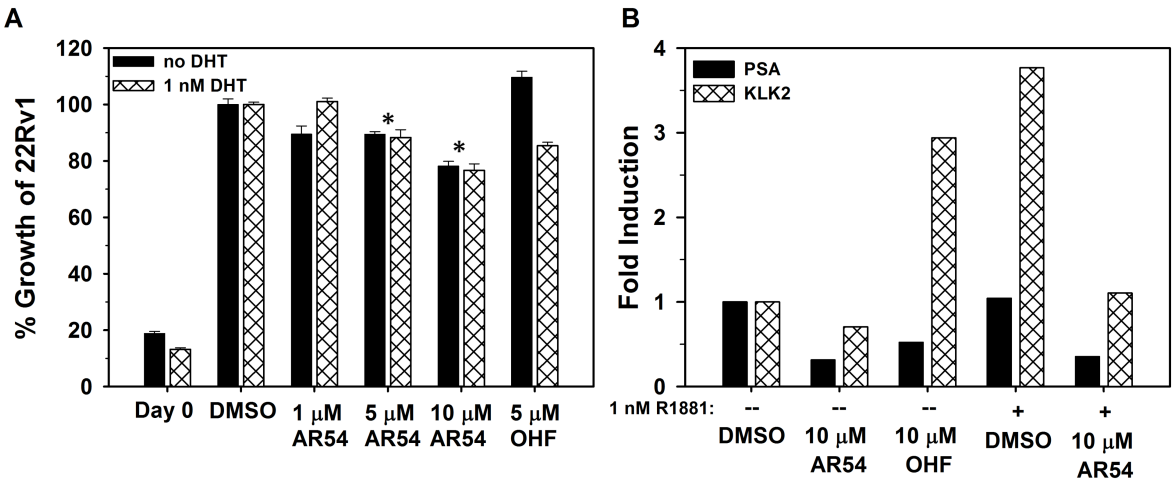
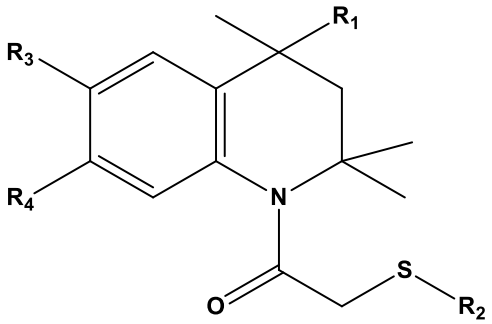


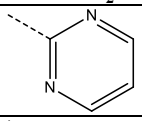
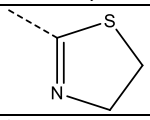
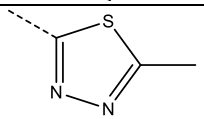
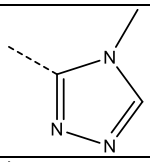
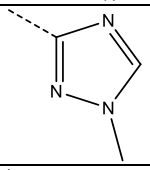
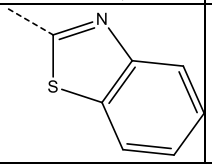

FIGURE 6.6. **AR54 suppresses proliferation of 22Rv1 cells and inhibits AR-mediated gene expression.** *A*, 22Rv1 cells were maintained for 4 days in 5% CD-FBS containing medium and plated at 5000 cells/well in 96-well plates. Cells were maintained without added androgens (no DHT) or with 1 nM DHT and the indicated concentrations of AR54 or hydroxyflutamide (OHF) and assayed using MTS after 5 days. Cell growth in DMSO after 5 days was set to 100% and data points represent the mean of 6 wells ± SEM. *B*, 22Rv1 cells were plated in 6-well plates and treated with indicated ligands and inhibitors for 24 h. PSA and KLK2 mRNA levels determined by qRT-PCR using β-actin to normalize. Significance of the differences between CPIC and the DMSO control was tested using Student's T-test and $p < 0.05$ when compared to the respective controls.

## SUPPLEMENTAL TABLES[3]

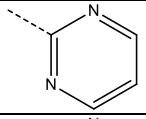
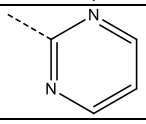
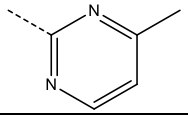
### Table 6S.1




**Group 1: Phenyl**

| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| AR54 | Phenyl |  | - - H | - - H |
| ARD61 | Phenyl |  | - - H | - - H |
| ARD55 | Phenyl |  | - - H | - - H |
| ARD53 | Phenyl |  | - - H | - - H |
| ARD03 | Phenyl |  | - - H | - - H |
| ARD42 | Phenyl |  | - - H | - - H |

**Group 2: No Phenyl, with methoxy**

| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| ARD30 | --H |  | $-OCH_3$ | - - H |
| ARD35 | - - H |  | - - H | $-OCH_3$ |
| ARD45 | - - H |  | $-OCH_3$ | - - H |

**Group 3: No Phenyl, with methyl**

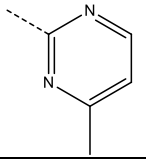
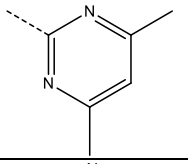
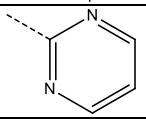
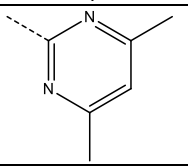
| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| ARD79 | --H |  | --H | - - H |
| ARD67 | - - H |  | --H | --H |
| ARD94 | - - H |  | $-CH_3$ | --H |
| ARD279 | - - H |  | $-CH_3$ | --H |

TABLE 6S.1. **AR54 and some structural analogues.** A common chemical scaffold of the compounds is shown, with $R_1$, $R_2$, $R_3$, and $R_4$ used for labeling the different substituents in the structure. Compounds were grouped into three categories based on a commonality indicated by the presence of phenyl, methoxy, methyl or none of the above groups at $R_1$, $R_3$ or $R_4$.

[3] The supplemental tables and figures in Chapter 6 were prepared by Khin Khin So H. Wu and are used here with her permission. These were part of an independent study conducted by Ms. Wu. I express my sincerest gratitude to Ms. Wu for her assistance and support in completing this work.

The actual structures of the analogues from Table 6S.1 are depicted below.

| Phenyl | | Methoxy | Methyl | |
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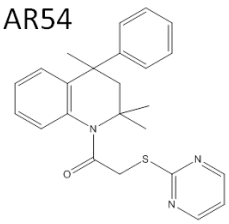
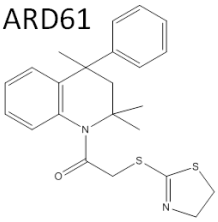
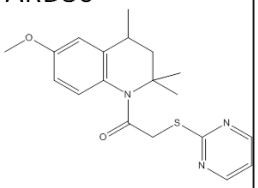
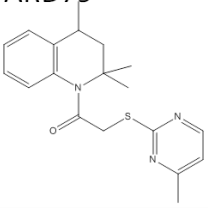
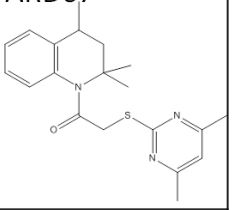
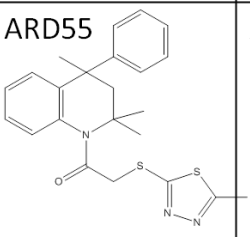
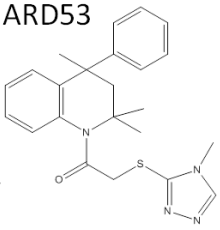
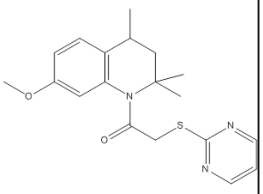
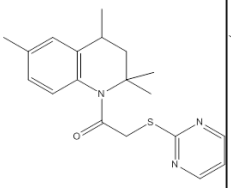
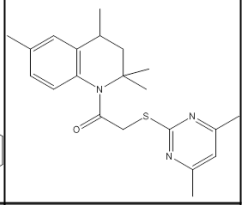
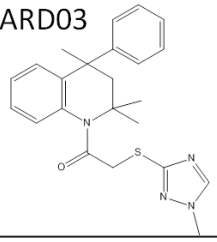
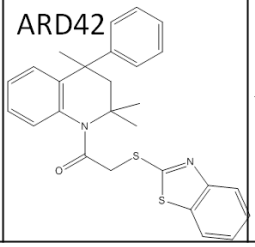
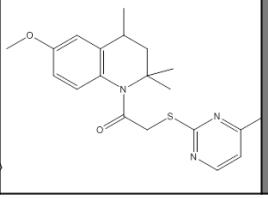
| AR54  | ARD61  | ARD30  | ARD79  | ARD67  |
| ARD55  | ARD53  | ARD35  | ARD94  | ARD279  |
| ARD03  | ARD42  | ARD45  | | |

**Structures of AR54 analogues.** These chemicals that are structurally related to AR54 were purchased from ChemBridge for structure-activity relationship studies.

**Table 6S.2**

| Compound | | LAPC4 MTS (Potency) | LNCaP MTS (Potency) | MCF-10A MTS (Toxicity) | PC-3 MTS (Toxicity) | T47D-KBLuc (Specificity) |
|---|---|---|---|---|---|---|
| GROUP 1 | **AR54** | $IC_{50}$ ≈ 1 -2 μM | $IC_{50}$ ≈ 4μM | Toxic at ≥20μM. (Drastic decline from 10 to 20 μM) | Toxic. 60% cell viability at 30μM. | 65% inhibition at 30μM |
| | **ARD61** | $IC_{50}$ ≈ 1.5μM | $IC_{50}$ ≈ 11.5μM | Very toxic at ≥10μM. | 10% cell viability at 20μM. | 54% inhibition at 30μM |
| | **ARD55** | $IC_{50}$ ≈ 1.5μM | $IC_{50}$ ≈ 12μM | Very toxic at ≥20μM. | 40% cell viability at 20μM. | 40% inhibition at 30μM |
| | **ARD53** | $IC_{50}$ ≈ 6.5μM | | | | |
| | **ARD03** | $IC_{50}$ ≥ 10.0μM | | | | |
| | **ARD42** | $IC_{50}$ ≈ 2.5μM | | 50% cell viability at ≥ 5 μM. | 55% cell viability at 20μM. | |
| GROUP 2 | **ARD30** | $IC_{50}$ ≈ 9μM | $IC_{50}$ > 15μM | No toxicity seen until 30μM. (85% cell viability) | Minimal toxicity. 80% cell viability at 30μM. | |
| | **ARD35** | $IC_{50}$ ≈ 10μM | | No toxicity seen up to 30μM. | | |
| | **ARD45** | $IC_{50}$ ≈ 9.5μM | | No toxicity seen up to 30μM. | | |
| GROUP 3 | **ARD79** | $IC_{50}$ < 1μM | $IC_{50}$ ≈ 14μM | 49% cell viability at 20μM. | 49% cell viability at 30μM. | 25% inhibition at 30μM |
| | **ARD67** | $IC_{50}$ < 1μM | $IC_{50}$ ≈ 7μM | 73% cell viability at 30μM. | 70% cell viability at 30μM. | 22% inhibition at 30μM |
| | **ARD94** | $IC_{50}$ ≈ 3.75μM | $IC_{50}$ > 15μM | No toxicity seen up to 30μM | 64% cell viability at 30μM. | 20% inhibition at 30μM |
| | **ARD279** | $IC_{50}$ ≈ 1μM | $IC_{50}$ ≈ 9.5μM | 42% cell viability at 30μM. | 46% cell viability at 20μM. | No inhibition of activity up to 30μM. |

TABLE 6S.2. **Properties of AR54 and analogues.** MTS assays were performed to measure cell growth and inhibitory effects of compounds in different cell-lines. LAPC-4 and LNCaP are prostate cancer cell lines. MCF-10A is an AR-negative breast cancer cell line, and PC-3 is an AR-negative prostate cancer cell line. A luciferase reporter assay was performed with stable

T47D-KBluc cells. The gray blocks indicate that the assay was not performed for that compound with that particular cell line, because the compound failed to show potential as an effective inhibitor.

**Figure 6S.1**

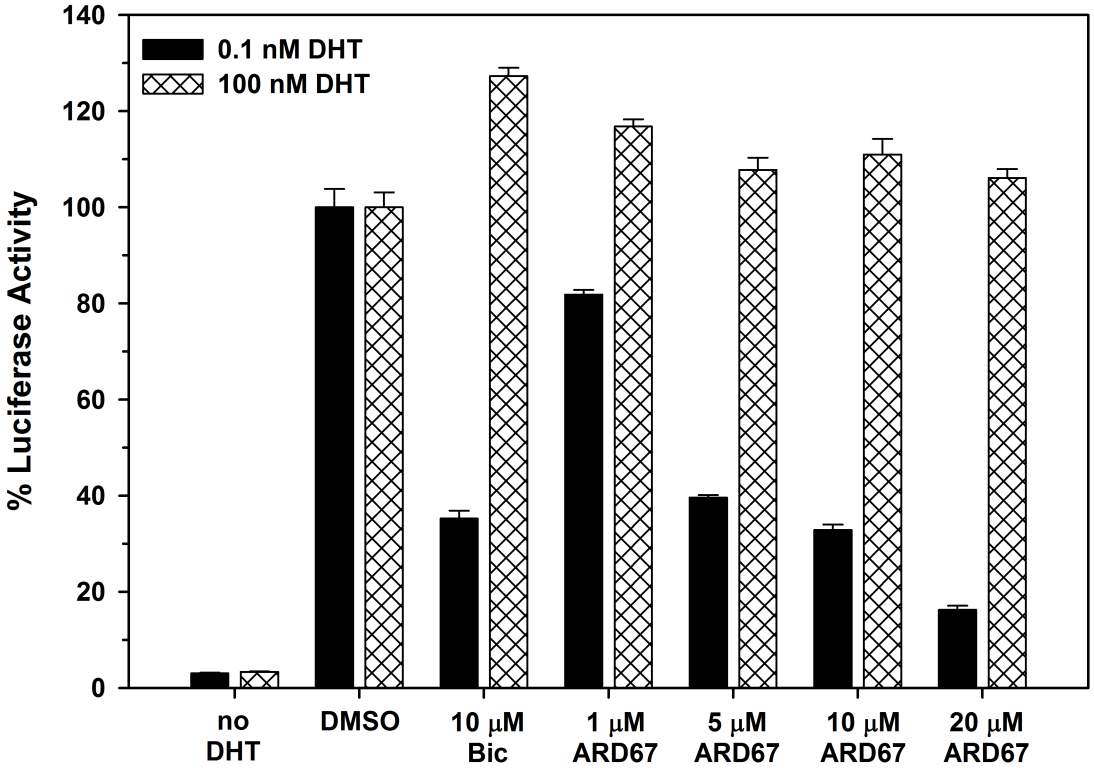



FIGURE 6S.1. **ARD67 is a competitive inhibitor of AR.** We analyzed the effect of DHT concentration on ARD67 inhibition of AR induced luciferase activity. HeLaA6 cells were seeded in 24-well plates and maintained for 24 h in medium containing 0.1 nM DHT (white bars) or 10 nM DHT (black bars) and the indicated concentrations of ARD67. Luciferase units of DHT-treated wells were set at 100%. Data represent the average of triplicate experiments ± SEM.

# CHAPTER 7
# DISCUSSION

Steroid hormone receptors play an important role in physiological development and in maintaining a healthy constitution. The significance of androgen and estrogen receptor action is emphasized by the fact that any deviation from their normal modus operandi can result in a range of chronic to morbid disorders. The androgen and estrogen receptors are unique among steroid receptors in their proliferative capacity; a trait that has serious ramifications in the context of prostate and breast cancer progression. For the past few years, our laboratory has been involved in efforts aimed at identifying and characterizing new small molecule inhibitors of androgen and estrogen receptor action. These inhibitors could then serve as starting points for the development of therapeutically relevant drugs and additionally be useful as probes to improve our understanding of steroid hormone receptor signaling. We performed a few moderate-to-high throughput screens to help us identify applicable chemical scaffolds. This document elaborates on screening methodologies and mechanistic studies performed to better understand the 'lead' chemicals we identified from each screen.

## High throughput screening

HTS is a repetitive procedure once the steps required for implementation are designed. But it requires a fair amount of initial groundwork before assembly into a fully feasible high-throughput screen. Since the screen for AR inhibitors was the first cell-based high throughput screen carried out on this campus, there was no template to follow. Every step in the screen was carefully planned and executed on a pilot scale before the actual screen. There were quite a few challenging hurdles before each segment of the screen was set up. For example, the liquid-handling systems could only dispense certain fixed volumes of compounds and we had to make sure to design the screen in the most 'pocket-friendly' way possible. These constraints challenged us to devise new non-traditional ways, especially for cell-based screening and analysis.

The first screen we set-up was a relatively easy biochemical assay. We tried to identify small molecules that interfere with the DNA binding function of full-length androgen receptor (AR). This fluorescence-based assay contained very few components and hence raised fewer

technical concerns. The most pertinent concern we had was regarding the intrinsic fluorescence activities of certain small molecules. These chemicals had to be eliminated as false positives, even though there was no telling whether any of them were real inhibitors. The screen had a robust Z'-factor value of 0.8, which makes it a very strong assay for HTS. We were able to execute the screen using 2.5 μM of each compound, a low concentration, in hopes of identifying only potent inhibitors. By comparing the results of the AR screen to similar screens performed using estrogen and progesterone receptors, we were able to eliminate most of the non-specific inhibitors. Everything seemed fine until we tested these chemicals in cell-based assays. With the exception of one, all the small molecules failed to suppress androgen-mediated luciferase expression, presumably due to their lack of cell permeability. We were unable to pursue the only viable compound identified by this screen due to lack of availability and challenging synthesis protocols involving explosives (1).

Our experience made us reevaluate our approach and we decided to set up a cell-based HTS of a much larger chemical library with the hope that individual hits will be identified with better potency, efficacy and specificity. A significant part of HTS success is dependent on the design and execution of the primary screening procedure. The more stringent and rigorous screens are rewarded with fewer false positives and false negatives. Cell-based assays combine the biological complexity of live cell responses with the scalability and process adaptation of HTS but it is an inherently challenging operation. The high degree of crosstalk between signaling pathways in a cell makes it biologically complex and increases potential sources of noise and variability. But on a positive note, it also makes the screen unbiased to a certain degree and allows of the identification of small molecules with different modes of action.

Many months were spent in validating the individual steps in the cell-based HTS screen. Just for comparison, the FAMA screen took one week for the primary screen with ~4 weeks of assay development. The cell-based HTS assay development took a few months to establish and the primary screen itself spanned several months. During the assay development phase, we designed a new way for performing cell-based screening as detailed in Chapter 3.

One major drawback of the screen was the increased incidence of false-positives, which in reality were cytotoxic chemicals. It would have been advantageous to couple the luciferase reporter-based primary screen to a toxicity screen in AR-negative cells. We tried to circumvent this issue by selecting a larger pool of compounds for secondary screening. Generally a hit rate

of 1-2 compounds/384-well plate is considered to be ideal after primary screening, but we selected about 5-6 compounds/plate.

The screen was implemented using a stable HeLa cell line that expresses luciferase enzyme robustly in response to androgens, working through the stably expressed AR. Although, it is known that most well-studied prostate cancer cell lines are unsuitable for HTS (LNCaP, attach poorly to wells while LAPC-4 cells have a long doubling time) it might still have been advantageous to use a prostate cancer derived cell line for the purpose of the primary screen. This might have ensured that the small molecules are able to work in signaling network and co-regulator environment mimicking actual prostate tissue.

The most common potential alternative to HTS is to identify hits using rational design or virtual screening. These approaches require significant structural information that is not currently available for much of AR. The N-terminal region of AR plays a critical role in transactivation. This unstructured region has been elusive to structural studies. Until the crystal structure for the AR amino-terminal domain is solved, it remains difficult to predict chemical scaffolds that could interact successfully at this domain and inhibit AR transactivation. Thus rational design and virtual screening are presently not feasible and HTS is the only realistic approach for identifying novel inhibitors that act outside the ligand binding domain of AR.

In spite of the reasonable success of our AR HTS, it remains puzzling that we found no specific AR inhibitor with potency in the nanomolar range, unlike the ER screen performed by others using the same set-up in different cells. Is ER easier to inhibit than AR? It is certainly possible, and what we observed in practice, but real proof for such a hypothesis is not available at this time. One point to note is the shorter and comparatively less active amino terminal domain of ER, which makes it a much more structured protein than AR. The flexible AR NTD constitutes more than half this protein and could be contributing to being harder to inhibit by requiring higher affinity small molecule binding.

**AR54**

We are only at the preliminary stages of lead development of this small molecule. Listed below are some experiments to shed light on the mechanism of action of AR54 and to enable us to tap into its long-term therapeutic potential, if any.

***Does AR54 directly bind AR? -*** As AR54 is not a competitive ligand, it is of utmost importance that we show conclusively whether or not it binds the androgen receptor. Recently, Anderson et al. used alterations in fluorescence emission patterns to help show that EPI-001 a small molecule AR inhibitor binds specifically to the AR N-terminal domain (2). Steady-state fluorescence emission spectra of full-length AR bound to AR54 could be performed if large amounts of purified androgen receptor are available. By analyzing fluorescence emission from tryptophan (λmax 343 nm) and tyrosine (λmax 305 nm) residues in the receptor, and by comparing the spectra resulting from solutions containing DMSO, AR54, or an inactive analogue of AR54, it is possible that we can deduce whether AR54 truly binds AR. Using synthetic chemistry, if a successful fluorescent analogue of AR54 is developed, it will be feasible to use that for binding studies (As demonstrated for EPI-001 in a talk by Dr. Marianne Sadar at the ENDO2011 Conference).

***How does AR54 decrease AR mRNA levels?-*** Experiments in LAPC-4 cells using actinomycin-D, an inhibitor of transcriptional initiation, have been unsuccessful so far. The concentrations of actinomycin-D used in these experiments were in the highest range possible without killing cells, and yet were incapable of reducing AR mRNA levels in the control treatments. AR54 at concentrations ≤10 μM are specific for AR mRNA suppression when compared to ERα. The major mechanisms to probe for would include transcriptional control by regulators at the AR gene level or translational repression and gene silencing resulting from microRNA action.

The long-term goal of this study is the development of potent small molecule inhibitors, capable of enhancing AR degradation at the molecular level that translates into increased survival in various models of prostate cancer.

**CPIC; a promising new inhibitor of AR action**

We aspire to advance this promising AR inhibitor or one of its putative analogues, from mechanistic studies in the laboratory through preclinical studies. Although discussed here, these long-term goals are beyond the scope of this thesis.

***Identify the site(s) on AR that interact with CPIC -*** We recently showed that CPIC robustly inhibits transactivation by the N-terminal AR-1-660. Based on our data, the $IC_{50}$ for CPIC inhibition of AR-1-660 is likely in the range of 200 nM. This inhibition is not due to non-specific

toxicity as transfections with GAL-VP16 in the same cell line treated with 10 μM CPIC show no inhibition. In addition to its actions at the N-terminus of AR, CPIC also exhibits properties consistent with action as a competitive inhibitor of androgen binding to full-length AR (Chapter 4). This suggests that the inhibitory effects of CPIC may be mediated through multiple regions of AR, or by inhibiting AR binding to DNA response elements. Competition assays with purified AR LBD can be used to determine whether CPIC retains the ability to exhibit a competitor phenotype without the AR N-terminus. Alterations in the fluorescence spectrum of expressed AR NTD, LBD and DBD can also be used to identify the site(s) on AR that interact with CPIC.

CPIC reduces the AR N/C interaction, presumably by interacting with the AR-NTD. One possibility is that CPIC binding induces an AR conformation that disfavors androgen binding and is inactive. Androgen binding induces an alternative active conformation that opposes the effect of CPIC. In this model, CPIC binding in the N-terminal region exhibits a "competitor phenotype"- even though it does not directly compete with androgens for binding in the ligand-binding domain. The major effect of this model is that in cases of CRPC when androgens are either absent or present at post-castration low concentrations, CPIC will be a considerably more effective inhibitor of AR action than it is in our early tests conducted at very high saturating concentrations of androgens. Alternatively, it remains possible that CPIC inhibits some unknown external factors (such as cytokines, kinases or growth factors), the activity of which might be crucial for AR transactivation in all cell types. Since CPIC is relatively specific for inhibition of AR and not other steroid receptors, this seems less likely.

***Determine the mechanism by which CPIC inhibits transactivation by constitutively active AR -*** It might be interesting to determine whether CPIC inhibits constitutive AR N-terminal activity in the same way as it inhibits full-length AR. These studies could include nuclear compartmentalization, DNA binding and coregulator recruitment, coimmunoprecipitation, RNAi knockdown, protein expression, mammalian two-hybrid assays, ChIP, microarrays and bioinformatics. They could establish whether CPIC uses the same mechanism to inhibit truncated and full-length AR, and whether N-terminal and full-length AR use the same pathway and coregulators to activate target genes.

***Lead optimization of CPIC using limited structure-activity relationships (SAR) -*** A dozen commercially available structural analogues of CPIC were evaluated as part of optimization studies. One small molecule PIC19.7 was identified to have partially activity in inhibiting AR

transactivation, while all the other analogues of this chemical family tested were inactive (data not shown). This limited structural data could serve as a guide for synthesis of CPIC derivatives with improved potency and specificity. Promising compounds that emerge from optimization can be evaluated for specificity in cell-proliferation studies using multiple AR and AR N-terminal positive and negative cell lines, tested for effectiveness against AR1-660 and constitutively active splice variants ARv$^{567}$es reported in CRPC. Microarrays studies or on a more ambitious note, multiplex gene expression studies (using platforms like the Luminex FlexGene LDA assay or L1000) can test for off-target effects and a genome-wide transcriptional profiling of CPIC treatment.

***Test CPIC in mouse xenograft models-*** The *in vivo* effectiveness of CPIC (or the best compound to emerge from optimization) has to be determined before we can ascertain that it has potential for use in human therapy. This could be tested in the androgen-dependent CWR22 human prostate cancer xenografts. There are no widely accepted xenograft models whose growth is driven by an AR splice variant. The CWR22 human prostate cancer xenograft resembles the majority of prostatic cancers. The tumor secretes PSA, regresses after castration and recurs as a palpable, growing, and ultimately fatal tumor.

In the long-term, CPIC might be suitable for further optimization and development as a potential therapeutic for aggressive late-stage prostate cancers resistant to current therapies.

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